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In vitro, evaluation of anti-mycobacterial active plant extracts against *Mycobacterium tuberculosis* using BACTEC mycobacteria growth indicator tube 960 automated system

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Abstract
The emergence of drug-resistant *Tuberculosis* has become a global health problem. Herbal drugs promise other potential options for *Tuberculosis* treatment. This study aimed to investigate the anti-mycobacterial activity of three plants: *Piper longum, Cressa cretica*, and *Calotropis gigantea.* The methanolic extract of these plants was tested against *Mycobacterium tuberculosis* H37Rv using the BACTEC MGIT-960 Automated system, and the minimum inhibitory concentration was determined. The extracts exhibited inhibitory activity against *Mycobacterium tuberculosis* H37Rv with *Piper longum* and *Cressa cretica* inhibiting at 125 µg/ml and *Calotropis gigantea* inhibiting at 250 µg/ml. However, the standard drugs isoniazid and rifampicin showed higher inhibitory activity against *Mycobacterium tuberculosis* at concentrations of 0.05 µg/mL and 0.12 µg/mL, respectively, compared to the crude plant extracts. This study highlights the anti-tuberculous potential of *Piper longum* and *Cressa cretica* as anti-mycobacterial agents. Nevertheless, further purification, molecular characterization and *in vivo* testing are necessary to evaluate their efficacy as novel anti-TB agents.

Keywords: Anti-mycobacterial, crude extracts, medicinal plants, *Mycobacterium tuberculosis*

1. Introduction
In 2023, a staggering 10 million new cases of *Tuberculosis* (TB) and 1.5 million TB-related deaths were reported globally. Additionally, there were 450,000 newly reported cases of drug resistance to rifampicin, a key TB medication [1]. Given this alarming situation, there is an urgent need to explore new anti-tubercular drugs that can be produced quickly, easily, and with fewer toxic side effects. One potential source of such drugs is herbal plant derivatives, which may offer lower chances of resistance and hepatotoxicity [2].
Traditionally, *Cressa cretica* (*C. cretica*) locally termed Rudravanti has been used as an anti-*Tuberculosis* agent, particularly for respiratory diseases, due to its bronchodilatory, antitussive, antibacterial, antipyretic, and analgesic effects [3]. It exhibits both bacteriostatic and bactericidal properties [3]. Another herbal medicinal plant, *Piper longum* (*P. longum*) locally termed as Pipli, is known for its efficacy in treating respiratory tract infections. Moreover, it has been found to enhance humoral immunity by increasing circulating antibody levels and antibody-forming cells [4]. The immunomodulatory activity of *P. longum* is believed to be a result of its combined effects on both humoral and cell-mediated immune responses [4]. *Calotropis gigantea* (*C. gigantea*), used in traditional medicine to treat infectious diseases like leprosy and TB, exhibits a wide range of beneficial properties such as antimicrobial, anticancer, anti-inflammatory, antidiabetic, antioxidant and larvicidal effects [5]. Numerous researchers worldwide, including in India, have investigated the inhibitory properties of medicinal plants against *Mycobacterium tuberculosis* (*Mtb*) [6, 7].
Study aimed to examine the *in vitro* antimycobacterial activity of methanolic extracts derived from these medicinal plants and explore their potential as therapeutic agents against *Mtb*.

2. Methodology
This study was conducted at the *Tuberculosis* containment laboratory (BSL-III facility) in the Division of Communicable Diseases of ICMR-National Institute of Research in Tribal Health, Jabalpur, India. Taxonomically verified plants were collected from the herbal garden of Jawaharlal Nehru Agricultural University, Jabalpur district of Madhya Pradesh, India. The nomenclature of the selected plants was compared to the TROPICOS database [8].

This study comes under a pre-experimental research study. Minimum inhibitory concentration (MIC) was determined using BACTEC Mycobacteria Growth Indicator Tube 960 Automated System

3. Material and methods

3.1 Preparation of Plant material

The collected plant materials were washed with tap water and then with distilled water 2-3 times. The washed plant material was shade dried, crushed in a mechanical grinder to make fine powder and stored at room temperature. Five grams of powdered material was used for extraction with 250 mL of 100% methanol as solvent. This mixture was continuously kept in the soxhlet apparatus at low temperature (15-25 °C) for 24-48 hours until the solvent appeared colorless. This concentrated extract was stored at 4 ºC in a desiccator for all further experiments. Stock solutions of crude extracts were prepared using Dimethylsulfoxide (DMSO) to a 70 mg/mL concentration following MGIT-960 protocol and sterilized through a syringe filter of 0.2 µm pore size [9].

3.2 Phytochemical Screening

Standard qualitative test procedures were conducted as described by Amita Pandey *et al*. (2014) to ascertain the presence of phytoconstituents. A 10 mg of the dry plant extract was dissolved in 10 mL of distilled water and methanol, respectively, to obtain separate extract solutions. These test solutions were utilized for phytochemical tests to identify various compounds such as tannins, flavonoids, alkaloids, steroids, etc. All reagents and chemicals employed in this experiment were procured from HiMedia, (Maharashtra, India).

3.3 *Mycobacterium tuberculosis* strain and culture medium

H37Rv (ATCC 25618) was used as a reference strain for the anti-mycobacterial assay, *Mtb* culture with DMSO (1.2%), Isoniazid (INH) at MIC99 (0.05 µg/mL) and Rifampicin (RIF) at MIC99 (0.12 µg/mL) were used as controls.

3.4 Inoculum and extract preparation

A 500µl of 1:5 diluted 0.5 McFarland bacterial suspension of *Mtb*-H37Rv was inoculated in MGIT 960 (BD, USA) tubes containing test compounds and controls. The inoculum was prepared from solid culture using Lowenstein–Jensen (LJ) slants, following the method described by Tulin *et al*. (2012). To provide a brief overview, the suspension was prepared in Middlebrook 7H9 broth from positive slant cultures and adjusted to a turbidity of 0.5 McFarland. A volume of 1 mL of the 0.5 McFarland H37Rv suspension was diluted in 4 mL of sterile saline [7].

The bacterial growth monitored according to the instructions provided by the manufacturer. Specifically, 100 µl of the extract was individually added to MGIT tubes containing *Mtb* and the tubes were then incubated at 37 °C in the BACTEC system. The growth units (GU-400) were monitored over a six-day period. As part of the MIC evaluations, a control was prepared by culturing a 1% bacterial suspension of 0.5 McFarland in the MGIT tube without any extract.

To determine the MIC of the extract, all the extract preparations were tested at two-fold decreasing concentrations, from 1 mg/mL to 0.12 mg/mL. We compared the growth units of the extract-containing samples to the growth units of the control (GU-400) and identified the lowest extract concentration that was equal to or lower than GU-400 as the MIC. After 42 days of incubation at 37 °C, the growth or inhibition of H37Rv was observed in both the extract-containing samples and the extract-free control and recorded the results accordingly.

4. Results

Qualitative phytochemical screening for phenols, terpenoids, sterols, tannins, flavonoids, alkaloids, carbohydrates, glycosides, and proteins was performed as described in Materials and Methods. The results of phytochemical screening revealed the presence of several bioactive compounds in the extract of *C. cretica*, including phenols, sterols, tannins, flavonoids, alkaloids, carbohydrates, glycosides, and proteins. In contrast, *P. longum* extract was found to be devoid of phenols, tannins, flavonoids, and glycosides. *C. gigantea* contains flavonoids, triterpenoids, phenolic compounds, proteins and sterols found in cardiac glycosides. However, it showed positive test results for sterols, alkaloids, carbohydrates, and proteins. A summary of the identified compounds in each plant is shown in Table 1.

Table 1: Qualitative Phytochemical Screening of the *Piper longum*, *Cressa cretica* and *Calotropis gigantea* Crude Extract

Name of metabolites	*Piper longum*	*Cressa cretica*	*Calotropis gigantea*
Phenols	-	+	+
Terpenoids	-	-	+
Sterols	+	+	+
Tannins	-	+	-
Flavonoids	-	+	+
Alkaloids	+	+	-
Carbohydrates	+	+	-
Glycosides	-	+	+
Protein	+	+	+
Anthraquinone	-	-	-

P. longum, *C. cretica*, and *C. gigantea* were tested at various concentrations. In the preliminary *in vitro* screening, the methanolic extracts of *P. longum* and *C. cretica* exhibited inhibitory effects on H37Rv with a MIC of 125 µg/mL each. *C. gigantea* demonstrated inhibition at 250 µg/mL but to a lesser extent than *P. longum* and *C. cretica*. All three methanolic extracts displayed growth inhibition against *Mtb*-H37Rv, with MICs ranging from 125 µg/mL to 250 µg/mL. In comparison, both INH and RIF demonstrated relatively higher inhibitory activity against *Mtb*-H37Rv, even at low concentrations of 0.05 µg/mL and 0.12µg/mL, respectively, when compared to the crude extract (Table 2).

Table 2: Susceptibility testing and minimum inhibition concentration (MIC) of three crude extracts of medicinal plants against *Mtb*-H37Rv using MGIT BACTEC 960 system

Name of Plants/drugs	Susceptibility activity (µg/mL)	MIC of the methanolic crude extracts(µg/mL)
Cressa cretica	< 150	125
Piper longum	< 150	125
Calotropis gigantea	< 300	250
Isoniazid (INH)	< 25	0.05
Rifampicin (RIF)	< 25	0.12

5. Discussion

According to the United Nations Conference on Trade and Development, more than 33% of medicines are derived from plants, and the World Health Organization has identified over 20,000 medicinal plants known for their therapeutic properties (World Health and Plants, 2006). In the context of TB treatment, previous studies have documented the use of various medicinal plants, such as *Acalypha indica*, *Adhatoda vasica*, *Allium cepa*, *Allium sativum*, *Acalypha indica*, and *Aloe vera* [10].

In the current study, the presence of phenols, sterols, tannins, flavonoids, glycosides, and alkaloids was observed in the extracts of *P. longum*, *C. cretica*, and *C. gigantea*. Among these compounds, alkaloids, terpenes, resin, monoterpenoids, sesquiterpenoids, and phenols have been identified for their anti-*Mtb* activity [11]. While *C. cretica* and *P. longum* have traditionally been used by tribal communities for various respiratory ailments, including TB [4, 11], there is currently no published evidence supporting their use as anti-tubercular agents. Furthermore, the existing literature on their anti-tubercular effects specifically related to proliferation is limited.

In this study the MGIT-960 system to assess the efficacy of plant extracts, while previous reports have mainly utilized the MGIT-960 method to determine the MIC of conventional anti-TB drugs [12]. Results revealed MIC values of 125 µg/mL for both *P. longum* and *C. cretica* against Mtb, while *C. gigantea* exhibited a MIC of 250 µg/mL. Notably, these MIC values for *P. longum* and *C. cretica* are being reported for the first time. The methanolic extracts of *P. longum*, *C. cretica*, and *C. gigantea* demonstrated significant antimycobacterial activity, which could be attributed to the active compounds present in these medicinal plants and their synergistic action.

The utilization of plant-derived compounds as anti-mycobacterial agents offers several advantages, including fewer side effects, higher patient acceptance due to their historical use, and reduced costs [10].

6. Conclusion

This study has revealed that *P. longum*, *C. cretica* and *C. gigantea* have the potential to be effective anti-TB drugs. However, these crude extracts and their active component needs to be purified. Their active principles should be characterized in both *in vitro* and *in vivo* studies to identify the clinical potential. The limitation of the study is that it was done on crude extract and hence the MIC is very high compared to the in-use anti-TB drugs.

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8. Conflict of Interests

None declared

9. Contributors

VT, JB and SR conceived the design of the study. VT, NP, and HT carried out the experiments. VT, JB and RS were involved in drafting and revision the manuscript. All the authors believe this to be honest and original work and have read and are responsible for the manuscript's content.

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11. Ethical clearance

The Institutional Ethical Committee (IEC) of ICMR–NIRTH, Jabalpur approved the study with a reference number NIRTH/IEC/539/2018 dated 5/06/2018

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