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Antimycobacterial activity of *Citrullus colocynthis* (L.) Schrad. against drug sensitive and drug resistant *Mycobacterium tuberculosis* and MOTT clinical isolates

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ABSTRACT

Ethnopharmacological relevance: *Citrullus colocynthis* (Cucurbitaceae), a folk herbal medicine and traditionally used natural remedy for tuberculosis in India has been studied to validate its antitubercular activity against drug sensitive and drug resistant (including multidrug resistant) *Mycobacterium tuberculosis* and Mycobacterium other than tuberculosis (MOTT) bacilli.

Materials and methods: Inhibitory and bactericidal activities of crude extracts, fractions and compounds of *Citrullus colocynthis* plant, consisting of aerial parts and ripe deseeded fruits were performed against the drug sensitive standard strain of *Mycobacterium tuberculosis* H37Rv (ATCC 27294), 16 drug resistant strains of *Mycobacterium tuberculosis* and two MOTT strains, using radiometric BACTEC 460TB system.

Results: Methanolic extract of ripe deseeded fruit of *Citrullus colocynthis* has shown good activity (MIC≤62.5 μg/ml), whereas among the bioactive fractions, FC IX demonstrated the best activity (MIC 31.2 μg/ml) against *Mycobacterium tuberculosis* H37Rv. Bioactive FC III, IX and X also inhibited 16 clinical isolates of *Mycobacterium tuberculosis* consisting of seven non-multidrug resistants, eight multidrug resistants, one extensively drug resistant and two of MOTTs with MICs in the range of 50–125, 31.2–125 and 62.5–125 μg/ml, respectively. Ursolic acid and cucurbitacin E 2-0-β-D-glucopyranoside were identified as the main biomarkers active against *Mycobacterium tuberculosis* H37Rv (MICs 50 and 25 μg/ml respectively), as well as against the 18 clinical isolates. FC III and FC IX showed better inhibition of drug resistant and MOTT clinical isolates. Minimal bactericidal concentrations of extracts, fractions and compound C-2 were ≥two-fold MIC values.

Conclusions: The study provides a scientific rationale for the traditional use of *Citrullus colocynthis* fruit in the treatment of tuberculosis. In addition, the study elucidates a broad spectrum antimycobacterial action of *Citrullus colocynthis* fruit, which can contribute to the development of improved preparation of an antitubercular natural drug for the treatment of drug resistant tuberculosis and MOTT infection as well.

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB), is one of the most dreadful infectious diseases that caused at; least 1.1 million deaths in the year 2010 (World Health Organization, 2011). The situation is likely to go worse with the emergence of multidrug resistant (MDR) and extensively drug resistant (XDR) strains. The increased incidences of, difficult to treat and diagnose TB like pulmonary and extra-pulmonary diseases, caused by *Mycobactrium*, other than tuberculosis (MOTT) (Shannon and Kasperbauer, 2008) are also a matter of concern for the clinicians.

The XDR TB is almost impossible to treat with the antitubercular drugs (ATD), currently available. The insufficient therapeutic arsenal, available for the treatment, necessitates the development of new anti-TB agent that is effective to drug resistant Mycobacteria and at the same time economical too.

Plants have long been used as a valuable source of anti-infective drugs (Cowan, 1999) wherein, 62–80% of population rely on traditional medicines for the treatment of common illness (World Health Organization, 2002). An ethnopharmacological approach where detailed observations and scientific validation of these traditional uses have given rise to many pharmaceutically relevant substances such as quinine from *Cinchona succirubra* (Rubiaceae) and colchicine from *Colchicum autumnale* ;(Colchicaceae) (Heinrich, 2001).

Citrullus colocynthis (L.) Schrad. (Cucurbitaceae), is an important and widely used plant in traditional medicine. Its use was well known to Greeks, Romans and the Arabs (Trease, 1976). It is a small herbaceous, trailing plant, with prostate or climbing stem. The fruits are smooth, spherical and mottled green when young,

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yellow when ripe and are known as "bitter apple", "colocynth", "vine-o-Sodom" or "tumba". Each part of the plant is very bitter. It is either cultivated or found wild in the warmer areas of Africa and Asia. In India, it grows wild in the warm and arid sandy tracts of Northwest, Central and Southern areas (Bhandari, 1995).

In India the plant is used by indigenous people as a natural remedy for bacterial infections including tuberculosis and other respiratory diseases.

Pharmacologically *Citrullus colocynthis* has been evaluated for its antimicrobial activity (Dallak et al., 2009; Memon et al., 2003; Najafi et al., 2010). However, the plant has not been explored scientifically for its antimycobacterial activity against tuberculosis causing *Mycobacterium tuberculosis* and non-tubercular respiratory infection causing Mycobacterium other than tuberculosis (MOTT). Therefore, this study was designed to validate the use of *Citrullus colocynthis* in the treatment of tuberculosis in the traditional medicine by evaluating the activity against drug sensitive and drug resistant *Mycobacterium tuberculosis* and MOTT. The study was further extended to identify and characterize the biomarkers responsible for the antimycobacterial activity, using bioassay guided fractionation with the idea of finding new antitubercular drugs.

Previously, the colocynth has been reported to contain cucurbitacins A, B, E, I, J, K, and L and their glycosides, iso-vitexin and its derivatives, iso-orientin and iso-saponarin (Maatooq et al., 1997; Nayab et al., 2006; Yoshikawa et al., 2007), which may be responsible for its activity.

2. Materials and methods

2.1. Mycobacterial strains

The drug sensitive standard strain of *Mycobacterium tuberculosis* H37Rv (ATCC 27294) as a reference strain, a total of 16 clinical isolates of *Mycobacterium tuberculosis* (MDAM 009-001–16) and two clinical isolates of MOTT (MDAM 009-0025–26) were included in the study. Out of 16 clinical isolates of *Mycobacterium tuberculosis* there were eight MDRs (50%), one XDR (6%) and seven non-MDRs (44%). MDRs were resistant to at least isoniazide (INH) and rifampin (RMP), the first line ATDs. XDR was resistant to the second line ATDs, capreomycin and ciprofloxacin in addition to isoniazide (INH) and rifampin (RMP). Further non-MDRs were mono- or multi-drug resistants (World Health Organization, 2003).

Recovery of all the clinical isolates was done from the pulmonary specimens of the patients in 7H12 Middlebrook broth (BACTEC 12B medium) containing ^{14}C-labeled palmitic acid as a substrate, using a BACTEC 460TB system (Becton-Dickinson Diagnostics Instruments Systems, Sparks, MD).

2.2. Plant materials

Plant material consisted of aerial parts (stem and leaves) and ripe fruits of *Citrullus colocynthis*, which was collected from Dist. Dausa Rajasthan, India during spring (mid-April to August). The plant was authenticated by taxonomist according to the "Flora and Fauna of Indian desert" (Bhandari, 1995). A voucher specimen (voucher no.–RUBL211309) has been deposited in the herbarium of the Department of Botany, University of Rajasthan, Jaipur, India.

2.3. Extraction, bioassay guided fractionation and compound isolation

The aliquots (50 g each) of shade dried and finely powdered aerial parts and deseeded fruits were taken from their main stocks and extracted with petroleum ether, chloroform, methanol and water (2×250 ml), in a Soxhlet apparatus for 72 h. The solvents were filtered and evaporated under vacuum (at 50 °C) to provide four crude extracts of aerial parts: petroleum ether (PCCA, 1.38 g), chloroform (CCCA, 3.31 g), methanol (MCCA, 6.67 g), and aqueous (ACCA, 4.11 g) and four crude extracts of fruits: PCCF (0.77 g), CCCF (4.17 g), MCCF (7.02 g), and ACCF (4.68 g). All the eight extracts were evaluated for their *in vitro* antimycobacterial activity against the pathogenic strain of *Mycobacterium tuberculosis* H37Rv (ATCC 27294). MCCF was found to be the most active and was re-prepared in bulk by extracting 2.5 kg powdered fruit from the previous stock, with methanol (2×2.5 l) using the same method and conditions mentioned earlier to give a dark brown semisolid mass (330 g). The extract was stored at −20 °C.

An aliquot (25 g) of MCCF was chromatographed over 650 g silica gel (60–120 mesh, Merck, India) in a glass column of 3.5 cm diameter and 1.5 m height. The column was eluted successively with the solvents (3×250 ml) of increasing polarity, in different combinations: petroleum ether pure (100%), petroleum ether: chloroform (80:20, 60:40, 40:60, and 20:80), chloroform pure (100%), chloroform:ethyl acetate (80:20, 60:40, 40:60, and 20:80), ethyl acetate pure (100%) and ethyl acetate:acetone (80:20, 60:40, 40:60, and 20:80). The fractions received, were then combined following similar analytic TLC pattern and concentrated to give 13 fractions (FC 1–13). Each fraction was primarily screened against *Mycobacterium tuberculosis* H37Rv at different concentrations and their MICs were determined. Anti-TB active fractions III and IX were further fractionated, compounds were isolated and purified by TLC and PTLC using the same solvent systems. Compound-1 identified as ursolic acid (154 mg) was obtained from FC III using solvent system: petroleum ether: acetone (4:1). Compound-2 identified as cucurbitacin E 2-0-β-D-glucopyranoside (196 mg) and Compound-3 as cucurbitacin I 2-0-β-D-glucopyranoside (44 mg), were obtained from FC IX using chloroform:methanol:ethyl acetate (3:3:4).

Three fractions, FC III (petroleum ether:chloroform, 40:60), FC IX (ethyl acetate 100%), FC X (ethyl acetate:acetone 80:20) and C-1, and C-2 were further evaluated against 16 drug resistant clinical isolates of *Mycobacterium tuberculosis* and two clinical isolates of MOTT.

The ^{1}H NMR and ^{13}C NMR spectra of all the isolated and purified compounds were recorded on JEOL AL-300 at 300.13 MHz using TMS as an internal control and $CDCl_3$ as a solvent. An elemental analysis was carried out using a Perkin-Elmer CHNS/O Analyzer 2400 and the mass spectra were recorded on an API QSTAR pulsar mass spectrometer. The structures of the compounds were confirmed by comparing with reference data from available literature.

2.4. Antimycobacterial activity evaluation

For the screening of the crude extracts, fresh stock suspensions of 40 mg/ml, of all eight extracts of *Citrullus colocynthis* aerial parts and fruits were prepared by macerating a requisite amount in a known volume of 3% dimethyl sulphoxide (DMSO). A two-fold serial dilution technique was used to make a set of concentrations for determining the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). For screening of the crude extracts a total of 0.1 ml of respective dilutions, were added to 4 ml of enriched 7H12 Middlebrook broth (BACTEC 12B vials) to achieve the desired concentrations in a high range (62.5–1000 μg/ml). The antimycobacterial activity evaluation of the fractions and compounds was started with the highest concentration $2 \times$ MIC of the methanol extract of fruits against *Mycobacterium tuberculosis* H37Rv. A set of concentrations in two-fold dilution from 15.6 to 125 μg/ml (prepared from stock-1; 20 mg/ml) and two additional intermediate concentrations 25 and