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IN-VITRO ANTIMYCOBACTERIAL ACTIVITY OF SELECTED INDIAN MEDICINAL PLANTS TO RESISTANT STRAINS OF *MYCOBACTERIUM TUBERCULOSIS*

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Keywords:

Glycyrrhiza glabra, Glycyrrhizin, *in vitro* antimycobacterial activity

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ABSTRACT: Tuberculosis (TB) remains a highly significant infection of microbial origin causing death of human in developing countries. The anti-tubercular drugs are less effective because of the emergence of multi-drug resistant strains and ineffective to extensively drug resistant strains of *Mycobacterium tuberculosis*. Plants are being an alternative source of anti-microbial compounds; the aim of this study was to investigate anti-mycobacterial potential of the few Indian medicinal plants and isolated compound from these to standard avirulent Mycobacterium tuberculosis H37Ra, standard virulent H37Rv and isoniazid resistant clinical isolates using Broth Microdilution Method. **Methods and Results:** Lyophilised ethanolic, aqueous and aqueous ethanolic extracts of four Indian medicinal plants viz. *Coleus amboinicus, Glychirrhiza glabra, Pongamia pinnata* and *Terminalia chebula* were screened for their *in vitro* antimycobacterial activity by Broth microdilution method (BMM) after 14 days of incubation at 37°C. The ethanolic extract of *G. glabra* was active against the tested strains of *M. tuberculosis* in primary screening with a MIC of 500µg/ml. The other extracts failed to exhibit inhibition at the tested concentrations. Glycyrrhizin a major and active constituent of the root of *G. glabra* was hence evaluated for its anitmycobacterial activity to the tested strains of *M. tuberculosis*. The isolated pure compound Glycyrrhizin exhibited a MIC value of 100µg/ml to the tested strains of M. tuberculosis. **Conclusion:** Our findings provide scientific support for ethno medical uses of *Glycyrrhiza glabra* to cure coughs and chest related ailments with the establishment of glycyrrhizin as a molecule for antimycobacterial activity and indicate a promising potential of this plant for the development of safer and efficacious anti-tuberculosis therapy.

INTRODUCTION: Tuberculosis (TB) is an infection of microbial origin causing death of human in developing countries transmitted by inhalation of aerosolized droplets of *Mycobacterium tuberculosis.* It affects almost all the organs of the body, the lungs being most commonly involved. Due to the emergence of multi-drug resistant (MDR) strains the anti-tubercular drugs have become less effective and ineffective due to emergence of extensively drug resistant (XDR) strains of *M. tuberculosis*.

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This has complicated the treatment and made TB more difficult to manage. Researchers around the world are increasingly turning their attention to traditional medicine in search of new and better antitubercular leads. Natural products play a significant role in the discovery and development of highly active antimycobacterial metabolites. The rhizome of *Glycyrrhiza glabra* Linn., commonly known as liquorice, is a reputed drug of Ayurveda. The rhizomes of *G. glabra* species have long been used worldwide as an herbal medicine and natural sweetener. Licorice is used to relieve 'Vata' and 'Kapha' inflammations, eye diseases, throat infections, peptic ulcers, arthritic conditions, and liver diseases in Indian Ayurveda system [1].

Aim:

The aim of this study was to investigate anti-mycobacterial potential of the plants and plant

derived compound using standard avirulent *M. tuberculosis* H37Ra, standard virulent H37Rv and isoniazid resistant clinical isolates using Broth Microdilution assay.

MATERIALS AND METHODS:

Bacterial Strains:

Standard strain H37Rv and INH resistant clinical isolates of *M. tuberculosis* were obtained from patients with pulmonary tuberculosis from the Tuberculosis Research Centre (NIRT), Chennai. Genotypic drug resistance of INH resistance isolates was detected by DNA sequencing analysis and mutations were reported in *kat G* gene and *inh A* gene of isolates [2].

Mycobacterium tuberculosis H37Ra (ATCC 25177) was obtained from ATCC. The cultures were maintained on Lowenstein-Jensen media slant at 37 °C. The details of the strains and isolates used for the study are presented in **Table 1**.

TABLE 1: CHARACTERISTICS OF STRAINS USED FOR TESTING THE ANTIMYCOBACTERIAL ACTIVITY

S.no	Strain/isolate	Drug susceptibility profile	*katG* genotype	*inhA* promoter genotype
1	H37Ra	Susceptible	Wild type	Wild type (absence of mutation)
2	H37Rv	Susceptible	Wild type	Wild type
3	RF 567	Resistant to SHOF	Mutant - R463L	Wild type
4	RF595	Resistant to HS	Mutant - R463L	Wild type
5	RF485	Resistant to H	Mutant - Insertion - 323 & 719	Wild type

Isolated compound:

The pure compound Glycyrrhizin was obtained through DST (TDT) Project from IIIM, Jammu.

Preparation of lyophilised extracts of medicinal plants for antimycobacterial activity to identify potential plant part/extract:

Collection of plant parts:

Healthy plant parts were collected air-dried in the shade at room temperature. The accuracy of the plant parts and family were ascertained with the Department of Plant Biology & Biotechnology, Presidency College (Aut), Chennai. **Table 2** shows the list of plants used in the study.

TABLE 2: LIST OF PLANTS USED FOR SCREENING

S. No	Name of the plant	Part used	Traditional use
1.	*Coleus amboinicus* Lour	Leaves	Cough, Chronic asthma, hiccup, bronchitis [3, 4]
2.	*Glycyrrhiza glabra* L.	Rhizome	Leprosy, consumption, asthma, bronchitis [3;] cough, expectorant , consumption, bronchitis, chest complaint, cough [5]
3.	*Pongamia pinnata (L.)*Pierre	Seed	Bronchitis and whooping cough [6]
4.	*Terminalia chebula* Retz	Fruit	Sore throat and chronic cough and asthma [6]

Preparation of plant extracts:

Four plants were chosen according to traditional use for treating respiratory diseases in the indigenous system of medicine in India and also for the treatment of tuberculosis based on literature. Crude extracts of the 4 medicinal plants were obtained. The healthy parts were surface sterilized with 70% ethanol. The parts were ground into powder and collected by sieving. Twenty grams (20 g) of the finely ground plant part was soaked with 100 ml Ethanol/sterile distilled water/50ml ethanol 50ml water (1:1) and stored overnight at 4ºC. Filtered and centrifuged to get clarified extract which were pooled together and filtered through a 0.22µm pore sized millipore filter.

The sterile extract was transferred to lyophilisation flask & freezed at -80^{0}C in deep freezer. The frozened extract was loaded to Lyophilizer. The lyophilised extract was stored in – 20^0 C till further studies.

Screening of lyophilised plant extracts/compound for Determination of Minimal Inhibitory concentration (MIC) by Broth Microdilution Method:

Screening test for detecting anti mycobacterial activity of plant extracts/compound was done by Broth Microdilution Method (BMM) as described by Chidambaram and Swaminathan 2013 [7]. The brief procedure includes.

1 mg of each plant extract/compound was dissolved in 100 µl of sterile distilled water and 900µl of 7H9 broth to make 1ml. It was mixed completely using a cyclomixer. Rifampicin and Isoniazid (INH) were used as reference durg controls and were prepared by dissolving in 100 µl water for INH and 100 µl DMSO for Rifampicin and mixed with 900µl Middle brook 7H9 broth medium. The stock solutions were sterilized through 0.20 µm sartorius single use filter.

Wells were filled with 0.1 ml amounts of Middle brook 7H9 broth, supplemented with oleic acid, albumin, dextrose and, catalase (ADC) enrichment.

200µl (100µg /100 µl) of the extracts/compound were serially diluted in Middle brook 7H9 broth. The initial concentration was 500µg / ml and the final concentration was 3.9µg/ml. Each well was inoculated with 5µl of 0.5 McFarland standard bacterial suspensions. As a growth control a well without antimycobacterial agents was also inoculated with of 5 µl of 0.5 McFarland standard bacterial suspension. 0.1 ml amounts of Middle brook 7H9 broth were added to all the wells and the plates were sealed and incubated at 37°C for 14 days in a moisturized incubator. Extracts/ Compounds demonstrating at least 90% inhibition in the primary screen were re-tested at lower concentrations in order to determine the actual minimum inhibitory concentration (MIC). The MIC was defined as the lowest concentration effecting absence of growth relative to drug controls.

RESULTS:

The anti-mycobacterial potency of the active plant extracts were determined by Broth Microdilution Method. Growth or inhibition of *M. tuberculosis* H37Ra, H37Rv, mono resistant and poly resistant isolates by Broth Microdilution method in extract/compound containing and in extract/compound free control wells after 14 days of incubation at 37°C were recorded. The preliminary *in vitro* screening of extracts revealed the lyophilized ethanolic plant extract of rhizomes of *Glychirrhiza glabra* to inhibit the tested strains with a MIC value of 500µg/ml. The other extracts failed to exhibit inhibition at the concentrations tested.

The isolated pure compound Glycyrrhizin was assayed by BMM for antimycobacterial activity to the tested strains which inhibited the tested strains *M. tuberculosis* H37Ra, H37Rv, mono resistant and poly resistant isolates with a MIC value of 100µg/ml.

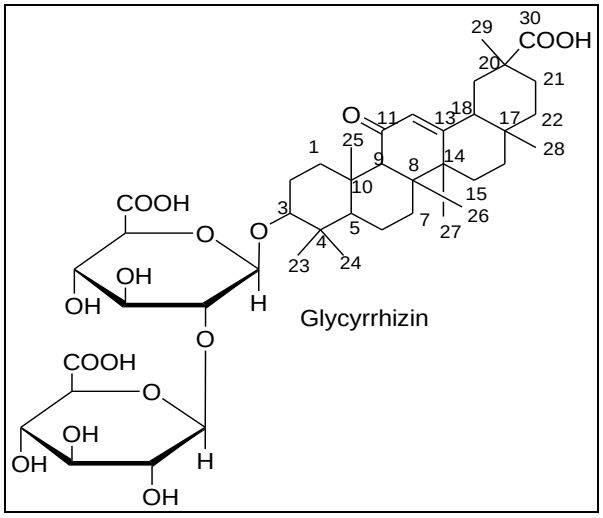

FIG. 1: CHEMICAL STRUCTURE OF GLYCYRRHIZIN

DISCUSSION: The advantages of using antimicrobial compounds from medicinal plants include fewer side effects, better patient acceptance due to long history of use, reduced costs and cultivability rendering them renewable in nature [8]. The present study was carried out to find out the *in vitro* antimycobacterial potential of few selected Indian medicinal plants based on ethno-medicinal uses. Antitubercular activity in *G. glabra* has been reported by Vivek K Gupta et *al*., 2008 in ethanolic extract at a MIC of 500µg/ml on H37Ra and H37Rv strains of *M. tuberculosis*. However the present observation has focused on the antitubercular activity of *G. glabra* extracts to isoniazid resistant clinical isolates in addition to standard avirulent H37Ra, virulent strain of H37Rv which are first time observations to the best of our knowledge. Hence we continued for activity of isolated active molecule.

Most earlier reported Indian studies on isolated compounds showed an antimycobacterial activity similar to the current study. Vivek K Gupta *et al.*, 2008 have isolated glabridin and hispaglabridin B. The antitubercular activity was exhibited by glabridin and was found to be at 29.16µg/mL against both the strains H37Rv and H37Ra. Ignacimuthu S and Shanmugam N 2010 studied

antimycobacterial activity of two compounds isolated from *A.vasica namely* Vasicine acetate and 2- acetyl benzylamine compounds and found Vasicine acetate and 2-acetyl benzylamine of *A.vasica* leaves inhibited *M. tuberculosis* multi-drug-resistant (MDR) strain at 200 μg/ml. Divya Lakshmanan *et al*., 2011 evaluated anti-tuberculosis activity of purified active molecule, identified as ethyl p-methoxycinnamate (EPMC) from rhizomes of *Kaempferia galanga* against Multidrug resistant isolate. EPMC was shown to inhibit MDR isolate (MIC 0.242–0.485 mM).

In the present study the isolated compound Glycyrrhizin was evaluated for its antimycobacterial activity to the tested standard and resistant strains by BMM and found to exhibit significant activity at 100µg/ml.

CONCLUSION: Our findings provide scientific support for ethno medical uses of *Glycyrrhiza glabra*, which is used in the treatment of chest related ailments with the establishment of glycyrrhizin as a molecule with appreciable antimycobacterial activity. Although the results from the present study are indicative that Glycyrrhizin has promising antimycobacterial activity, as this molecule is known to be a single drug with a potentiality to inhibit avirulent H37Ra, Virulent sensitive H37Rv, INH monoresistant, INH polyresistant strains further *in vitro* screening of this molecule for its efficacy to MDRTB is needed. Further studies on the mechanism of its action and structure and activity relationship (SAR) may also be estimated for it to be a better drug candidate in future.

CONFLICT OF INTEREST: We declare that we have no conflict of interest.

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