EVALUATION OF ANTI – TUBERCULAR ACTIVITY DIRECTLY FROM VERSA TREK MYCO BOTTLES USING *WRIGHTIA TOMENTOSA* ALCOHOLIC EXTRACTS

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Summary

The objective of the present study is to evaluate the antitubercular activity directly from Versa TREK myco bottles against Mycobacterium tuberculosis (MTB) and Mycobacterium other than tuberculosis (MOTT) using the plant based ethanolic leaf & bark extracts of Wrightia tomentosa in different doses, in order to combat the emergence of multi drug resistant tuberculosis caused by allopathic drugs. Test compounds I-VI (50mg, 100mg, 200mg) in three different doses each with leaf and bark extracts, and a separate control (drug free medium) were evaluated invitro against M.tuberculosis H37Rv using Middle brook 7H9 broth as the nutrient medium.For the susceptibility testing, Versa TREK system combines a liquid culture medium, a growth supplement and a specific concentration of ethanolic leaf and bark extract with a detection system that automatically incubates and continuously monitors culture bottles inoculated with isolates of M.tuberculosis and M.species other than tuberculosis from various specimen sources. The appearance of growth, if any was observed from 3rd day onwards. The results obtained were much more promising against non-tuberculous mycobacterial infections (NTM) rather than typical tuberculosis. The leaf extract of W. tomentosa (100mg) has proved to be useful against MOTT, as the growth identified (43.1hours) is comparatively larger than the control (11.9hours). Further investigation is needed to isolate the specific pure component and make an approach of effective target in combating NTM infections of hospitals and clinical labs.

Key words: Wrightia tomentosa, Typical, Atypical, Versa TREK system, Middlebrook 7H9 broth.

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Introduction

Eighty percent of the world's population relies on medicinal plants for their primary health care. Such herbal medicines are easily available, cheaper, time tested and considered safer than some of the modern synthetic drugs **(1)**. Tuberculosis (T.B) is a chronic respiratory disease resulting in 2-3 million deaths every year around the world **(2)**. The currently available medications show serious side effects like hepatotoxicity (isoniazid), damage to auditory nerve (streptomycin) & thrombocytopenic purpura (rifampicin) **(3)**.The emergence of multidrug resistant TB has further complicated complicated the therapy **(4)**. The World Health Organization declared TB a global health emergency in 1993, and the stop TB partnership developed a global plan to stop tuberculosis aiming to save 14 million lives between 2006 and 2015.

Mycobacterium tuberculosis is the bacterium that causes most cases of tuberculosis **(5)**. Other mycobacteria such as Mycobacterium bovis, Mycobacterium africanum , Mycobacterium canetti, and Mycobacterium microti can also cause tuberculosis ,but these species do not usually infect healthy adults **(6)**. The primary cause of TB, Mycobacterium tuberculosis (MTB) is an aerobic bacterium that divides every 16 to 20 hours, an extremely slow rate compared with other bacteria, which usually divide in less than an hour **(7)**. The M.tuberculosis complex includes three other TB-causing mycobacteria: M.bovis, M.africanum and M.microti. The first two only very rarely cause disease in immuno competent people. On the other hand although M.microti is not usually pathogenic, it is possible that the prevalence of M.microti infections has been under estimated **(8)**. Other Known pathogenic mycobacteria include Mycobacterium leprae, Mycobacteraum avium and M.kansasii. The last two are part of the non tuberculous mycobacterium (NTM)group .Non tuberculous mycobacateria cause neither TB nor leprosy, but they do cause pulmonary diseases resembling TB **(9)**.TB requires much longer periods of treatment to entirely eliminate mycobacteria from the body **(10)**.

People with latent infections are treated to prevent them from progressing to active TB disease later in life.The Centers for Disease Control and Prevention (CDC)notified health care professionals of revised recommendations against the use of rifampin plus pyrazinamide for treatment of hospitalization and death from liver injury associated with the combined use of these allopathic drugs **(11)**. However, in tropical areas where the incidence of atypical mycobacteria is high, exposure to nontuberculous mycobacteria gives some protection against TB **(12)**. Hence, a study was designed to target the latent tuberculosis using the plant based ethanolic leaf and bark extracts of Wrightia tomentosa in various doses against mycobacterium tuberculosis (Typical) and mycobacterium species other than tuberculosis (Atypical) by Versa TREK rapid culture system.

Wrightia tomentosa Roem . & Schult, family Apocynaceae, is widely distributed at an altitude of 600m in the Himalayas . A novel isoflavone, wrightiadione isolated from the plant possess cytotoxic activity against murine P388 lymphocytic leukemia cell line **(13)**. The root –barks are found to be useful in snake bite and scorpion –stings **(14)**. The butanol extract of the plant was reported to exhibit anti-microbial activity **(15)**. The ethanolic bark & leaf extract of Wrightia tomentosa possesses significant anti –allodynic effects **(16)** and antihyperglycemic activity **(17)** in streptozotocin induced diabetic rats.

Materials and Methods

I.Plant material

Wrightia tomentosa was procured from the hills of Yercaud forest, Salem district of Tamilnadu and authenticated by an acknowledged Botanist, Mr. Dhiravia Doss of the Research Department of Bharathidasan University, Tiruchirapalli, Tamilnadu, India and the voucher specimen was deposited there after at Bharathidasan University.

II. Extraction

The leaves and bark of Wrightia tomentosa were dried at room temperature and reduced to a coarse powder. The powdered materials (leaves and bark) were subjected to qualitative tests for the identification of various phyto constituents like alkaloids, glycosides, steroids,terpenoids and flavanoids. Then the powder was subjected to soxhlet extraction with benzene, chloroform, alcohol (90%) and water separately for 72 hours at a temperature of 50-60^{o}C. The extracts were concentrated & the solvent was completely removed. They were freeze dried and stored in the vacuum dessicator. Further, the ethanolic extracts of leaf and bark in different doses were used for antitubercular activity invitro against M. tuberculosis H37Rv and M. species other than tuberculosis (MOTT) in middle brook 7H9 broth media.

III. Methodology

1. Versa TREK myco susceptibility kit:

Rapid detection of M. tuberculosis and its susceptibilities are critical for effective patient management. For the drug extract susceptibility testing of M. tuberculosis, the versa trek system **(18)** combines a liquid culture medium (versa trek myco broth), a growth supplement (versa trek myco GS) and a specific concentration of ethanolic leaf & bark extract with a detection system that automatically incubates and continuously monitors culture bottles inoculated with isolates of M. tuberculosis & M. species other than tuberculosis from various specimen sources.

2. Protocol followed:

Both typical and atypical mycobacterial susceptibility test for assessing anti tubercular activity on leaf and bark extract of Wrightia tomentosa using Versa- trek rapid culture system was carried out at Doctor's Diagnostic center R & D Labs, Tiruchirappalli, Tamilnadu, India. Test compounds I-VI in different doses of both leaf & bark were evaluated invitro against M. Tuberculosis H37 Rv using Middle brook 7H9 broth as the nutrient medium containing ADC growth supplement **(19-21)**. Similarly, the same set of test compounds were also evaluated against M. species other than tuberculosis for atypical myco bacterial susceptibility testing.

3. Isolate preparation:

The source for isolate preparation was ESP myco seed bottle. A#1 Mc Farland equivalent (using sterile distilled or deionized water) was created with organisms from a growth in Middle brook 7H9 broth . In to a single versa trek myco bottle, 0.5ml .volume from above cell suspension and 1.0 ml GS (Growth Supplement) was aseptically injected and vortex the bottle thoroughly.

A connector has been attached aseptically and entered in to Versa trek instrument, incubated until bottle signals positive. Remove the bottle and then the connector. Vortex bottle vigorously for 1-2 minutes. The inoculum was prepared aseptically. Seed bottle must be used within 72 hours. The seed bottle was diluted (1:10) using sterile distilled or deionized water to obtain the final inoculum. 0.5 ml of this dilution was used to inoculate the sixteen Versa trek mycobottles used in the susceptibility test for both typical and atypical mycobacteria.

4. Myco susceptibility drug preparation:

Test compounds I-VI in different doses (50mg, 100mg & 200mg) were selected for ethanolic leaf extract and ethanolic bark extract (each extract possess 3 doses). All the test compounds were dissolved in minimum amount of dimethyl sulphoxide (4200 µl) and then diluted with 7H9 broth to get the desired concentration (500µl). To each of the tubes, 0.01 ml of freshly prepared inoculum of M. tuberculosis H37 Rv (matched to 0.5 Mc farland standard) was added. Dilutions of vehicle control (drug free) were treated similarly.

5. Incubation:

Each myco bottle was labelled with drug ID and concentration as sample access number. The bottle stoppers were disinfected with alcohol and aseptically added 1.0 ml of Versa Trek Myco GS (growth supplement) to all bottles. Aseptically added 0.5 ml of each rehydrated and diluted drug (500µl) to the appropriate bottle. The whole set was incubated **(22)** at 37^{o}c and the appearance of growth if any was observed from 3^{rd} day onwards. All experiments were performed in duplicate.

6. Interpretation of results:

For susceptibility testing using the Versa Trek /ESP. system, a test isolate was interpreted **(23)** as being susceptible or resistant to a drug based on the following formula: No growth of myco bacterium species with the specific anti-mycobacterial drugs for more than 3 days, after the growth in drug free culture (ie.control) considered as susceptible. Isolation of myco bacterium species in a drug-containing bottle on or before 3 days of control positivity was considered to be resistant.

Results

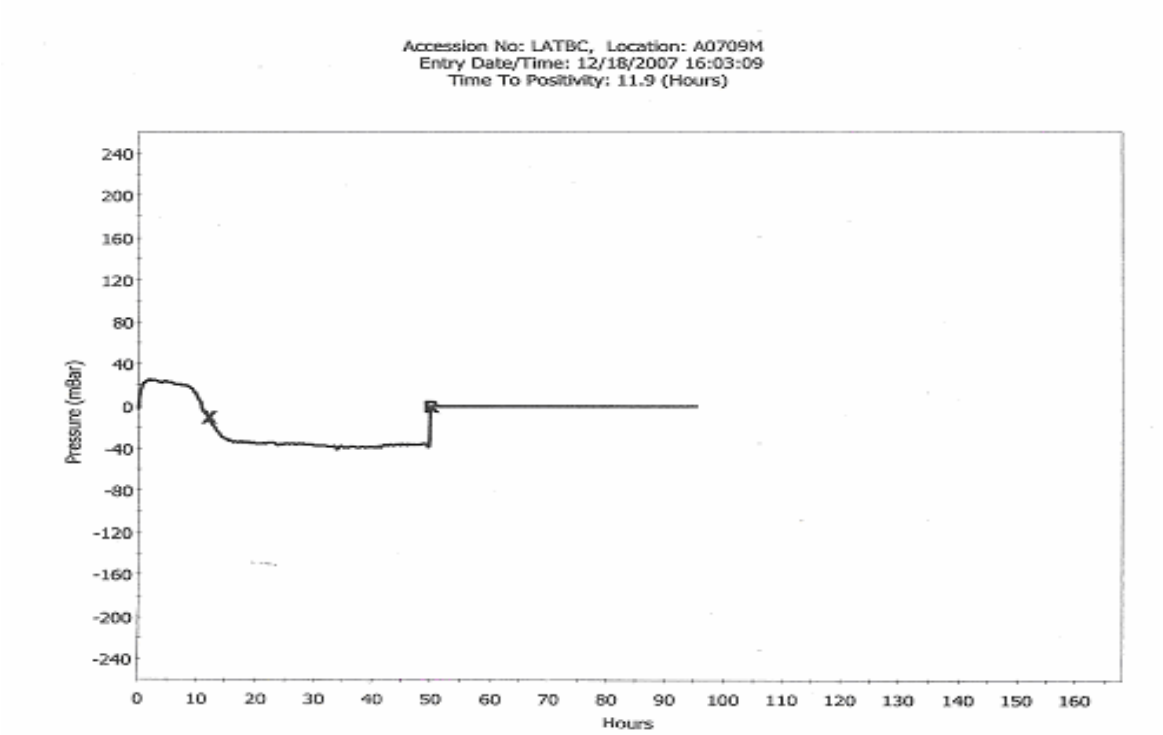
Tuberculosis are the most serious of the world's deadly diseases, and the search for new drug leads is an urgent need due to the emergence of drug –resistant strains of mycobacteria. This is the first report on the invitro activity of Wrightia tomentosa against M.tuberculosis and M.species other than tuberculosis. The results of both the typical & atypical mycobacterial susceptibility test for the leaf and bark extract of Wrightia tomentosa using versa trek rapid culture system were summarized in table 1 and table 2. Among the leaf extracts tested against typical mycobacterium, all the extracts in different doses (50 mg, 100 mg, 200 mg) showed the presence of mycobacterium species in a drug-containing bottle before 3 days of control positivity and hence are considered to be resistant. Higher doses of extract reduce the colony of mycobacterium species (evidenced from Table 1) as compared with lower doses of leaf extract. Similarly all the bark extracts are resistant with the isolation of mycobacterium species in drug containing bottle before 59.9 hours of control positivity. The time for positivity in hours was mainly due to microbial metabolism with the release or absorption of gases and they are subsequently interpreted with Versa Trek windows software version 5.2.9.6(service pack 2). Some of the compounds times for positivity are clearly evidenced from the following graph.

Table (1)

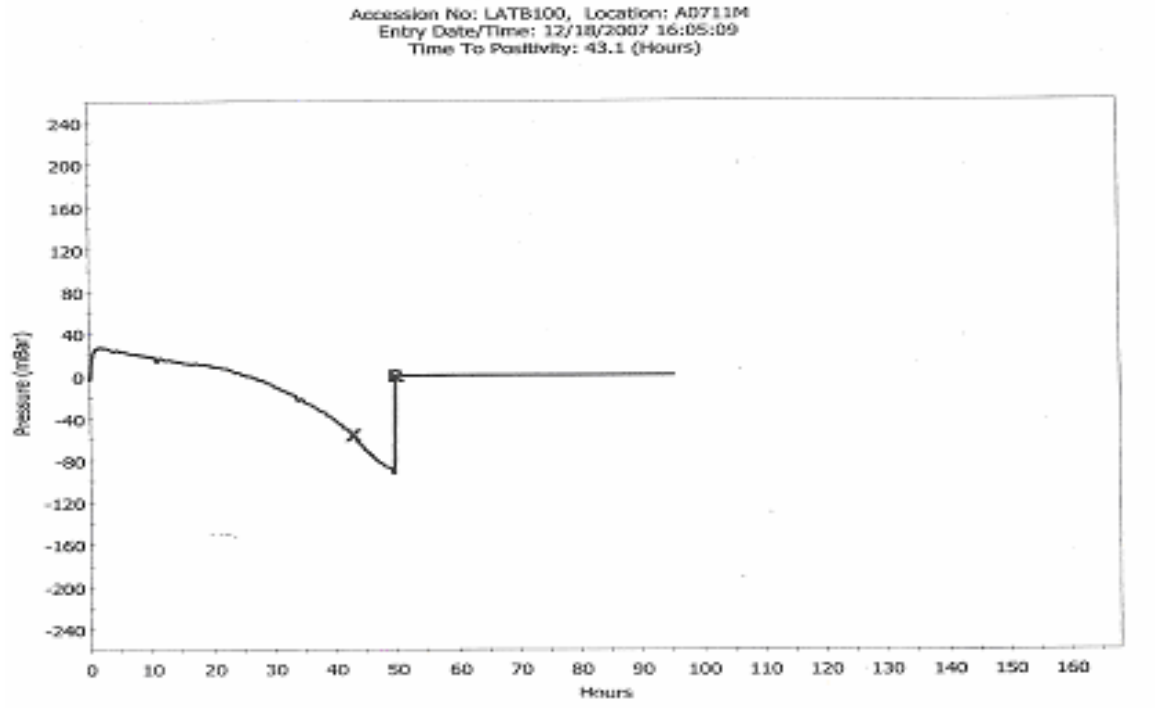
TYPICAL MYCOBACTERIAL SUSCEPTIBILITY TEST FOR THE LEAF & BARK EXTRACT OF *WRIGHTIA TOMENTOSA*

S.No	Type of sample	Sample access number	Concentration of the sample used	Days /Hours of Identifiable Growth	Status
1	Ethanol leaf extract	LMTB50	50 mg	Growth identified in 16.6 hours	Resistant
2	Ethanol leaf extract	LMTB 100	100 mg	Growth identified in 14.6 hours	Resistant
3	Ethanol leaf extract	LMTB 200	200 mg	Growth identified in 22.6 hours	Resistant
4	Leaf control	LMTBC	Drug- free medium	No Growth identified for more than 72.hours	_
5	Ethanol bark Extract	BMTB 50	50 mg	Growth identified in 14.6 hours	Resistant
6	Ethanol bark Extract	BMTB 100	100 mg	Growth identified in 16.6 hours	Resistant
7	Ethanol bark Extract	BMTB 200	200 mg	Growth identified in 12.6 hours	Resistant
8	Bark control	BMTBC	Drug free medium	Growth identified in 59.9 hours	_

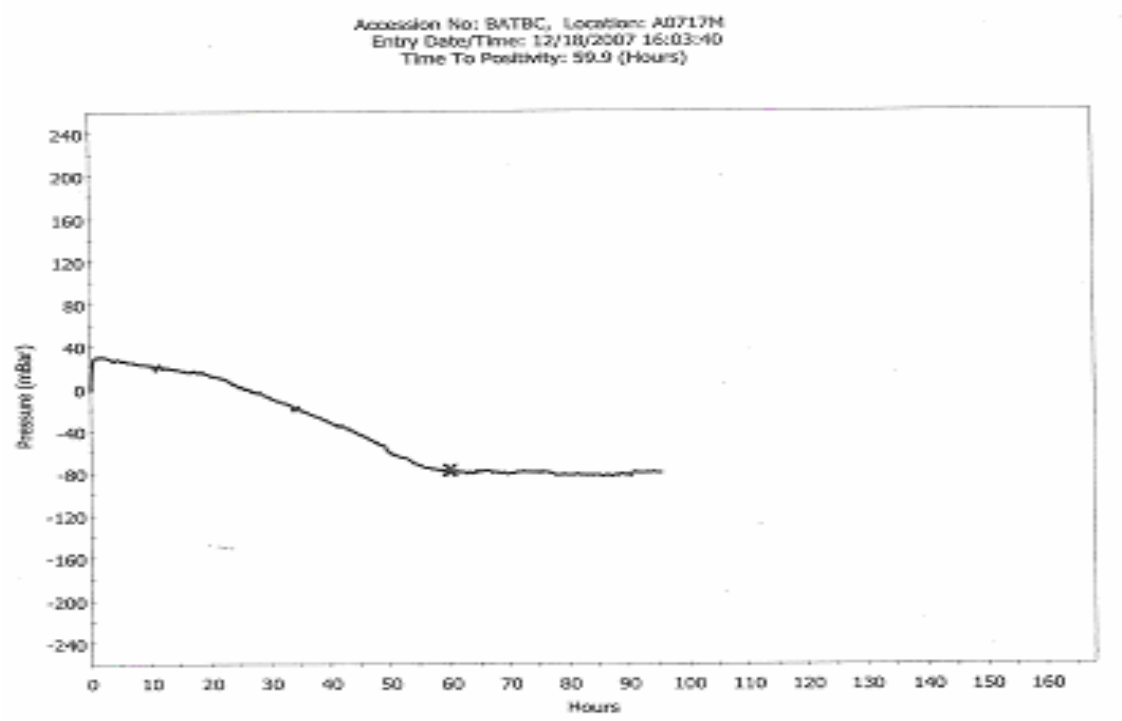
Control: Mycobacterium tuberculosis without the given compound
Susceptible: No growth in specific drug for >72hrs after control growth positivity
Resistant: Growth of Mycobacterium species in specific drug on or before control positivity.



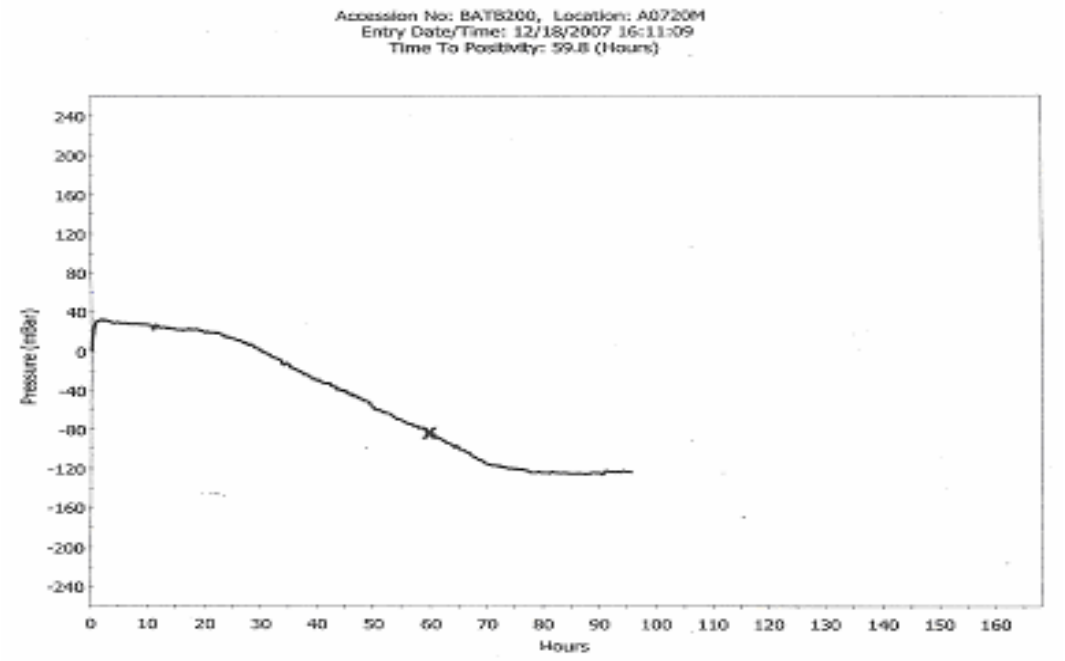
CONTROL GRAPH OFLEAF(DRUG FREE MEDIUM) AGAINST NON-TUBERCULOUS MYCOBACTERIAL INFECTION



TIME POSITIVITY GRAPH OF W.TOMENTOSA LEAF EXTRACT(200mg) AGAINST NON-TUBERCULOUS MYCOBACTERIAL INFECTION



CONTROL GRAPH OF BARK (DRUG FREE MEDIUM) AGAINST NON-TUBERCULOUS MYCOBACTERIAL INFECTION



TIME POSITIVITY GRAPH OF W.TOMENTOSA BARK EXTRACT(200mg) AGAINST NON-TUBERCULOUS MYCOBACTERIAL INFECTION

Table 2

ATYPICAL MYCOBACTERIAL SUSCEPTIBILITY TEST FOR THE LEAF & BARK EXTRACT OF *WRIGHTIA TOMENTOSA*

S.No	Type of sample	Sample access number	Concentration of the sample used	Days /Hours of Identifiable Growth	Status
1	Ethanol leaf extract	LATB 50	50 mg	Growth identified in 19.9 hours	Resistant
2	Ethanol leaf extract	LATB 100	100 mg	Growth identified in 43.1.hours	Resistant
3	Ethanol leaf extract	LATB 200	200 mg	Growth identified in 22.7.hours	Resistant
4	Leaf control	LATB C	Drug free medium	Growth identified in 11.9 hours	----
5	Ethanol bark extract	BATB 50	50 mg	Growth identified in 13.8 hours	Resistant
6	Ethanol bark extract	BATB 100	100mg	Growth identified in 14.6 hours	Resistant
7	Ethanol bark extract	BATB 200	200mg	Growth identified in 59.8 hours	Resistant
8	Bark control	BATB C	Drug free medium	Growth identified in 59.9 hours	----

Control: Mycobacterium species other than tuberculosis (MOTT) without the given compound.
Susceptible: No growth in specific drug for >72 hrs after control growth positivity.
Resistant: Growth of mycobacterium species in specific drug on or before control positivity.
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In connection with atypical mycobacterial susceptibility testing, leaf control (drug free) shown remarkable growth in 11.9 hours, whereas the leaf drugs tested (50mg, 100mg & 200mg)have shown growth in 19.9 hours, 43.1 hours and 22.7 hours respectively (Table 2). Eventhough the leaf extracts withstand time for sustained growth as compared with control, all the leaf extracts in different doses are considered to be resistant by considering 3 days growth time period. This 72 hours protocol standard is especially for FDA approved antitubercular drugs for clinical therapy. Among the bark extracts tested against atypical mycobacterium species, all the extracts in different doses (50 mg, 100 mg & 200 mg) are considered to be resistant .The bark control took 59.9 hours as time to positivity, whereas the bark extract of higher dose (200 mg) took 59.8 hours as time to positivity.This clearly indicates that the potency of bark control was almost similar with that of the 200 mg bark extract.

Culturing of Typical Mycobacterium (small vial) & Non-tuberculous Mycobacterium (large vial)

Versa TREK Rapid culture system for Drug susceptibility testing against Typical & Atypical mycobacteria

Discussion

The above results clearly indicated that all the leaf extracts and bark extracts of Wrightia tomentosa in different doses (50mg, 100 mg & 200 mg) doesn't possess significant anti-tubercular activity against Mycobacterium tuberculosis by typical mycobacterium susceptibility testing.

Non-tuberculous mycobacterium (NTM) infections are becoming a major concern for hospitals and medical clinics around the world. Non tuberculous mycobacterium, also known as atypical tuberculosis (Atypical TB) or Mycobacterium other than tuberculosis (MOTT), is a bacteria that is found in water, including hot tubs and showers, some domestic and wild animals, and soil. One of the most common forms of NTM infections found in humans is Mycobacterium avium complex (MAC). This is a primary cause of respiratory disease in humans and is a leading cause of death in HIV/AIDS patients.

Our results are much more promising against NTM infections (Table 2) rather than typical tuberculosis. Among the extracts tested against NTM infections, the leaf extract of Wrightia tomentosa (100mg) has proved to be extremely useful against mycobacterium species other than tuberculosis as the growth identified with the extract (43.1hours) is comparatively greater than the control (11.9 hours). This is mainly due to the presence of alkaloids & flavanoids as active constituents.

Further investigation is required to isolate the responsible pure component and make an effective approach of targeting NTM infections. Testing of rapidly growing species of mycobacteria against anti-bacterial agents has been shown to have some clinical utility. The methods used to perform this testing also need to be further validated.

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