

TARGETED DRUG IDENTIFICATION AND SUSCEPTIBILITY TESTING OF TYPICAL MYCOBACTERIUM TUBERCULOSIS DIRECTLY FROM VERSA TREK MYCObOTTLES WITH TROUBLE SHOOTING APPROACH OF EXTRACTS & ISOLATES FROM *WRIGHTIA TOMENTOSA*

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Summary

The objective of the present investigation is to assess the drug susceptibility testing of Mycobacterium tuberculosis directly from Versa TREK mycobottles with trouble shooting technique for the extracts & isolates from the herbal *Wrightia tomentosa* and thereby identifying the most potent targeted drug with its structure elucidation. Four test compounds in different doses (100mg & 200mg each from leaf & bark) were dissolved in minimum amount of dimethyl sulphoxide (4200µl) and then diluted with 7H9 broth to get the desired concentration (500µl). To each of the tubes, 0.01ml of freshly prepared inoculum of Mycobacterium tuberculosis H37 Rv (matched to 0.5 Mcfarland standard) were added. The tubes were disinfected with alcohol and 1.0ml of versa trek myco growth supplement were added aseptically & the whole set was incubated at 37°C for the presence / absence of growth from 3rd day onwards. The same procedure was followed for control by excluding the drug extracts in the sample tube. The extracts tested were identified with gram positive cocci and gram negative bacilli by Gram staining examination, lead to invalid results. Hence, a trouble shooting made to eliminate the cocci and bacilli by treating the extracts & isolates with U.V. radiation sterilization for 15 mts consecutively for 3 days. The extracts and isolates tested after sterilization had promising antitubercular effects. Among the results identified, one of the test isolate (Test compound VII-EBPCF₆₀ ; 100% Ethyl acetate- column effluent) was found to be highly susceptible as there was no growth identified even after 38.1 days of incubation. The maximum activity of the test compound VII is due to the presence of flavanoids, confirmed by TLC analysis. Further clinical investigation (in-vivo) is needed to explore the drug's potency.

Key words: *Wrightia tomentosa*, Mycobacterium tuberculosis, Versa TREK system, Isolate, Sterilization, Lead identification, NMR spectroscopy.

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Introduction

Tuberculosis (T.B) is a chronic respiratory disease resulting in 2-3 million deaths every year around the world (1). The currently available medications show serious side effects like hepatotoxicity (isoniazid), damage to auditory nerve (streptomycin) & thrombocytopenic purpura (rifampicin) (2). The emergence of multidrug resistant TB has further complicated the therapy (3).

Mycobacterium tuberculosis is the bacterium that causes most cases of tuberculosis (4). It 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 (5). TB requires much longer periods of treatment to entirely eliminate mycobacteria from the body (6).

Natural products have been our single most successful source of medicines. Each plant is like a chemical factory capable of synthesizing unlimited number of highly complex and unusual chemical substances whose structures could otherwise escape the imagination forever. Although clinical trials and experiments involving whole animals are important in natural product screening but because of financial, ethical and time limitations, importance of invitro screening is gaining popularity (7). Our objective is to study the drug susceptibility testing of typical mycobacterium tuberculosis directly from *Wrightia tomentosa* with plant based extracts and isolates obtained from leaf and bark of *Wrightia tomentosa* and thereto to elucidate the final structure of potent lead to combat tuberculosis.

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 (8). The root – barks are found to be useful in snake bite and scorpion –stings (9). The ethanolic bark & leaf extract of *Wrightia tomentosa* possesses significant anti –allodynic effects (10) with no observable signs of toxicity (11) and antihyperglycemic activity (12) in streptozotocin induced diabetic rats. The alcoholic extract of *Wrightia tomentosa* dried bark was reported to exhibit markedly high anti-oxidant activity (IC₅₀ value of 75.0 µg/ml from DPPH radical scavenging assay), suitable for prevention of human disease (13). The butanol extract of the plant was shown to have anti-microbial activity against both gram positive and gram negative organisms (14). The leaf extract (100 mg) of *Wrightia tomentosa* has proved to be extremely useful against non-tuberculous mycobacterium (NTM) infections (15), which are becoming a major concern for hospitals and medical clinics.

Based on the anti-microbial potency from leaf & bark extracts, a study was designed to evaluate the anti-tubercular activity against the slow growing pathogenic *Mycobacterium tuberculosis* with the extracts and isolates of the same herbal *W.tomentosa*. We assessed the anti-tubercular activity with extracts of 2 doses from leaf and bark and found that the test was invalid, as bacterial contamination was identified by Gram staining examination. Furthermore, a trouble shooting approach was made to

overcome the bacterial contamination with U.V. radiation sterilization for 3 days and thereafter the extracts and isolates were tested against *Mycobacterium tuberculosis* to identify the most potent isolate having utmost anti-tubercular effect.

Materials and Methods

I. Plant material

The leaves and bark of *Wrightia tomentosa* were collected 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 and Isolation

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 (650 gm) was subjected to Soxhlet extraction with benzene, chloroform and ethanol separately for 72 hours at a temperature of 50-60°C. The extracts were concentrated & the solvent was completely removed by Rotary vacuum evaporator (Buchi).

The concentrated ethanol leaf extract (3 gm) & bark extract (2.2 gm) were taken in a china dish separately and heated continuously on a water bath by gradually adding ethanol in small portion with constant stirring till desired consistency was obtained. Silica gel (for Column chromatography, 30-70 mesh size) was then added (weighed quantity 32.82 gm for leaf extract & 25 gm for bark extract) slowly with continuous mixing with steel spatula till desired consistency of the mixture was obtained. It was air-dried and larger lumps were broken to get a smooth free flowing mixture.

Two columns of 5.0 ft. length and 16 mm of internal diameter were taken and dried. The lower end of the column was plugged with absorbent cotton wool. The column was clamped and fitted in vertical position on a stand. The column was then half-filled with hexane. Silica gel was then poured in small portions and allowed to settle gently until the necessary length of the column was obtained. The dried silica gel slurry containing the ethanol extract of leaf and bark were poured in the columns separately and then eluted successively with different solvents, in the order of Ethyl acetate: hexane (5:5), ethyl acetate: hexane (7.5:2.5), ethyl acetate, ethyl acetate: ethanol (9:1), ethyl acetate: ethanol (8:2), ethyl acetate: ethanol (7:3), ethyl acetate: ethanol (6:4), ethyl acetate: ethanol (5:5), ethyl acetate: ethanol (4:6), ethanol and then with ethanol: water (9:1), ethanol: water (8:2), ethanol: water (7:3), ethanol: water (6:4), ethanol: water (5:5) and water. The fractions collected in the conical flask were marked. The marked fractions were subjected to TLC to check homogeneity of various fractions. Chromatographically identical various fractions (having same R_f values) were combined together and concentrated. They were then crystallized with suitable solvent systems.

Elution of leaf drug in column with ethyl acetate, i.e. (fraction 3) yielded dark green amorphous powder, R_f : 0.78 (Ethyl acetate: pyridine: acetic acid: water:62:21:6:11) for steroids was designated as ELPCF₃. Similarly, elution of leaf drug in column with ethyl acetate-ethanol (4:6), i.e. (fraction 9) yielded greenish black amorphous powder, R_f : not clear for alkaloids, flavonoids & steroids, was designated as ELPCF₉.

Elution of bark drug in column with ethyl acetate, i.e. (fraction 3) yielded green amorphous powder, R_f : 0.52 (Ethyl acetate: formic acid: acetic acid: ethyl methyl ketone: water:50:7:3:30:10) for flavonoids and was subsequently designated as EBPCF₃. Similarly, elution of bark drug in column with ethyl acetate-ethanol (7:3), i.e. (fraction 6) yielded greenish yellow amorphous powder, R_f : 0.48 (Ethyl acetate: formic acid: acetic acid: ethyl methyl ketone: water:50:7:3:30:10) for flavonoids and was designated as EBPCF₆. Hence two isolated components (ELPCF₃; ELPCF₉) from leaf and two components (EBPCF₃; EBPCF₆) from bark along with extracts in two different doses were selected for drug susceptibility testing against typical *Mycobacterium tuberculosis* H37 Rv using Versa Trek rapid culture system.

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 (16) has combined a liquid culture medium (versa trek myco broth), a growth supplement (versa trek myco GS) and a specific concentration of ethanolic leaf & bark extracts including pure isolates with a detection system that automatically incubates and continuously monitors culture bottles inoculated with isolates of *M. tuberculosis*.

2. Protocol followed:

The typical mycobacterial susceptibility test for the assessment of anti-tubercular activity on leaf and bark extract along with isolates of *Wrightia tomentosa* was carried out at Doctor's Diagnostic Center R & D Labs, Tiruchirappalli, Tamilnadu, India. Initially 2 doses from each extracts were taken as test compounds (100 mg, 200 mg) and designated as compound I (Leaf 100 mg), II (Leaf 200 mg), III (Bark 100 mg) & IV (Bark 200 mg). They were evaluated invitro against *M.tuberculosis* H37 Rv using Middlebrook 7H9 broth as the nutrient medium containing ADC growth supplement (17-19).

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 six Versa trek mycobottles used in the susceptibility test for typical mycobacterium tuberculosis.

4. Myco susceptibility drug preparation:

Test compounds I-IV in different doses (100mg, 200mg each from leaf and bark) 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) were 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 (**20**) at 37°C and the appearance of growth if any was observed from 3rd 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 (**21**) 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. 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).

Results

The results of test compounds I-IV of typical mycobacterial susceptibility testing were shown in Table (1).

Table (1)
TYPICAL MYCOBACTERIAL SUSCEPTIBILITY TEST FOR THE LEAF & BARK
EXTRACTS OF WRIGHTIA TOMENTOSA

S.No	Type of sample	Sample access number	Concentration of the sample used	Days /Hours of Identifiable Growth	Status
1	Test Compound I-Ethanol leaf extract	LMTB100	100 mg	Growth identified in 14.6 hours	Test Invalid*
2	Test Compound II-Ethanol leaf extract	LMTB 200	200 mg	Growth identified in 22.6 hours	Test Invalid*
3	Leaf control-MTB with no drugs	LMTBC	Drug free medium (500µl sterile distilled water)	No growth identified for more than 72 hours	–
4	Test Compound III-Ethanol bark extract	BMTB 100	100 mg	Growth identified in 16.6 hours	Test Invalid*
5	Test Compound IV-Ethanol bark extract	BMTB 200	200 mg	Growth identified in 12.6 hours	Test Invalid*
6	Bark control-MTB with no drugs	BMTBC	Drug free medium (500µl sterile distilled water)	Growth identified in 59.9 hours	–

* Bacterial contamination was identified by Gram staining examination

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.

The above results have shown the invalid status of test compounds due to probable bacterial contamination within the samples. Gram positive cocci and gram negative bacilli were identified in the samples by Gram staining examination. Hence a trouble shooting approach was made to remove the contaminants & further tested against typical Mycobacterium tuberculosis.

Trouble shooting approach:

In order to overcome the presence of gram positive cocci and gram negative bacilli, U.V.radiation sterilization were done for all the extracts & isolates for 15 minutes. After three days of treatment, we have cultured the compound to rule out the contamination. No organisms were grown, even after 72 hours of aerobic incubation. Hence the test compounds (I-IV) and the pure component isolates (VI-VIII) were found to be free from microbial contamination. The same set of procedure was followed for the test compounds I-VIII as stated in methodology and the appearance of growth, if any was

observed from 3rd day onwards. The results obtained were highly promising due to trouble shooting using ultraviolet radiation sterilization. All the results obtained were tabulated in Table (2).

Table (2)
TYPICAL MYCOBACTERIAL SUSCEPTIBILITY TEST FOR *WRIGHTIA*
***TOMENTOSA* ETHANOLIC EXTRACT & PURE ISOLATE**
(U.V. Treated: 15 minutes for 3 consecutive days)

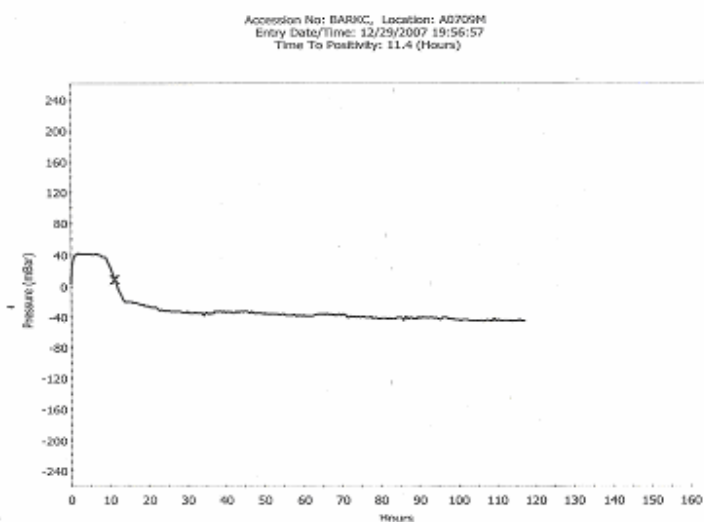
S.No	Type of sample	Sample access number	Concentration of the sample / 4200µl DMSO	Drug loaded (µl)	Days /Hours of Identifiable Growth	Status
1	Ethanol leaf Extract (Test compd.I)	LTB 100	100 mg	500	Growth identified in 16.3 hours	Resistant
2	Ethanol leaf Extract (Test compd.II)	LTB 200	200 mg	500	Growth identified in 59.9 hours	Resistant
3	Ethanol leaf Pure isolate-3 (Test compd.V)	ELPCF G-60	60 mg	500	Growth identified in 19.9 hours	Resistant
4	Ethanol leaf Pure isolate-9 (Test compd.VI)	ELPCF ₉ -60	60 mg	500	Growth identified in 17.9 hours	Resistant
5	Leaf control	LEAFC	Drug free medium	500 (Sterile distilled water)	Growth identified in 15.5 hours	—
6	Ethanol bark Extract (Test compd.III)	BTB 100	100 mg	500	Growth identified in 15.8 hours	Resistant
7	Ethanol bark Extract (Test compd.IV)	BTB 200	200 mg	500	Growth identified in 59.8 hours	Resistant
8	Ethanol bark Pure isolate-3 (Test compd.VII)	EBPCF-60	60 mg	500	No growth identified even after 38.1 days of incubation	Susceptible
9	Ethanol bark Pure isolate-6 (Test compd.VIII)	EBPCF-100	100 mg	500	Growth identified in 19.8 hours	Resistant
10	Bark control	BARKC	Drug free medium	500 (Sterile distilled water)	Growth identified in 11.4 hours	—

Control: Mycobacterium tuberculosis without the given compound

Susceptible: No growth in specific drug for >72hrs after control growth positivity

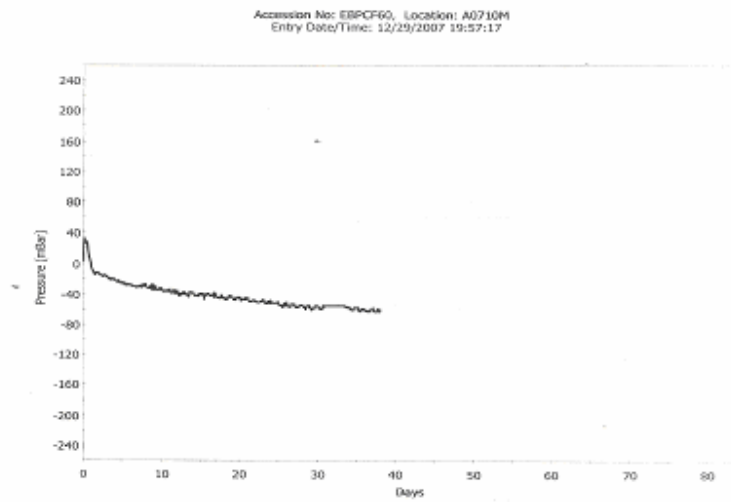
Resistant: Growth of Mycobacterium species in specific drug on or before control positivity.

Among the leaf extracts tested against typical mycobacterium, Test compounds I, II, V and VI showed growth in 16.3 hours, 59.9 hours, 19.9 hours and 17.9 hours whereas the leaf control shown remarkable growth in 15.5 hours respectively. In comparison with earlier studies of Table (1), U.V radiation sterilization had an impact for delayed time of positivity for the test compounds I and II. Even though the leaf extracts (I & II) and isolated compounds (V & VI) withstand time for sustained growth as compared with control, all the test compounds of leaf (I, II, V & VI) are considered to be resistant by considering 3 days growth time period. This 72 hours protocol standard is especially for FDA approved anti-tubercular drugs for clinical therapy. Some of the test compounds time for positivity was clearly evidenced with the following **Graphs** attached herewith.

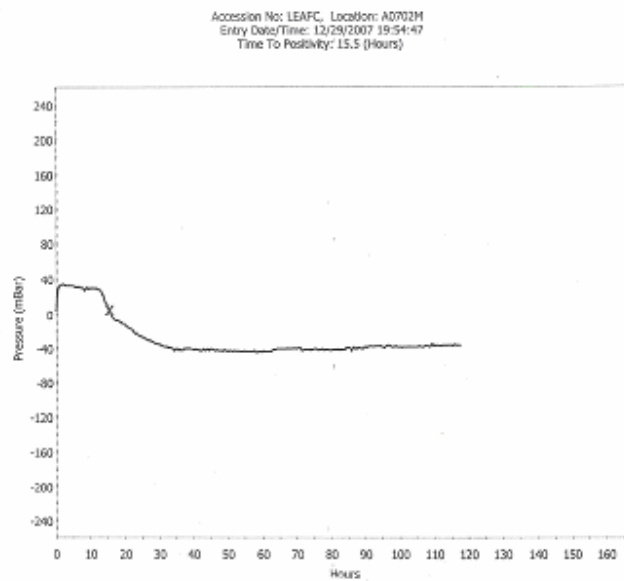


**CONTROL GRAPH OF BARK (DRUG FREE MEDIUM)
AGAINST TYPICAL MYCOBACTERIUM
TUBERCULOSIS**

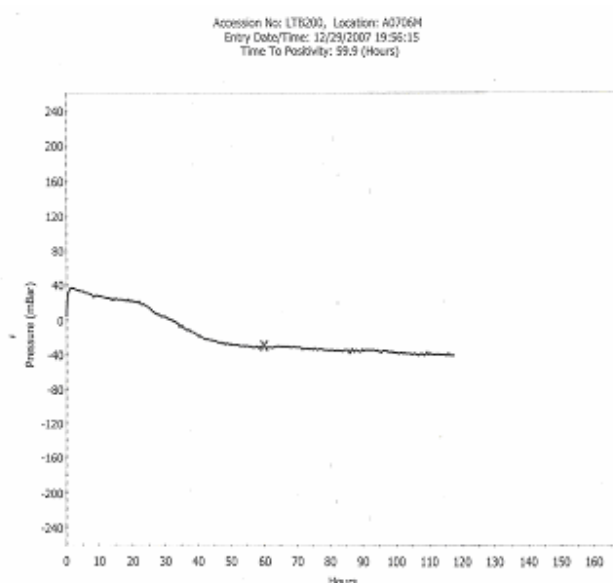
Among the bark extracts tested against typical mycobacterium species, Test compounds III (BTB 100), IV (BTB 200) & VIII (EBPCF-100) are considered to be resistant with the time for positivity in 15.8 hours, 59.8 hours and 19.8 hours respectively. The bark control took 11.4 hours as time to positivity. One of the pure component isolate from bark, i.e., Test compound VII (EBPCF₃-60) was found to be highly susceptible as there was no growth identified even after 38.1 days of incubation (Maximum days: 42).



**TIME POSITIVITY NULL RESPONSE GRAPH OF
W. TOMENTOSA BARK ISOLATE (EBPCF3) AGAINST
MYCOBACTERIUM TUBERCULOSIS**



**CONTROL GRAPH OF LEAF (DRUG FREE
MEDIUM) AGAINST TYPICAL MYCOBACTERIUM
TUBERCULOSIS**



TIME POSITIVITY GRAPH OF *W. TOMENTOSA* LEAF EXTRACT (200 MG) AGAINST TYPICAL *MYCOBACTERIUM TUBERCULOSIS*

Structural elucidation of most active pure isolate (EBPCF₃-60):

Isolated pure component (EBPCF₃) from running flash column was proved to be more active against typical *Mycobacterium tuberculosis*. Hence, its final structure was confirmed by ¹H-NMR, ¹³C-NMR, ¹H-¹H COSY and HMBC spectral datas. Preliminary phytochemical screening and TLC results have revealed that the pure component (EBPCF₃) was basically a flavonoid class of compound.

General experimental procedure: ¹H-NMR and ¹³C-NMR spectra were measured using a Bruker DRX 300 instrument with DMSO-d₆ as solvent (300.13 MHz for ¹H and 75.47 MHz for ¹³C). The COSY & HMBC (in DMSO-d₆) experiments were performed on the same instrument. ¹H-¹H-Correlated Spectroscopy (COSY) spectra were acquired with 1.0 sec. relaxation delay, 1562.500 Hz spectral width in both dimensions. The Heteronuclear multiple bond correlation (HMBC) spectra were obtained with 1.0 sec. relaxation delay, 1562.500 Hz spectral width in F₂ and 131.082 Hz spectral width in F₁.

Discussion

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.

The above results clearly indicated that one of the pure component fraction isolated from bark extract (Test compound VII-EBPCF₃-60mg) through column chromatography, (Fraction-3; 100% Ethyl acetate) possess the maximum anti-tubercular activity against typical *Mycobacterium tuberculosis* by Versa TREK rapid culture system directly from

mycobottles. EBPCF₆₀ doesn't show time for positivity even after 38.1 days of incubation and proved to be highly susceptible with the trouble shooting approach of using U.V. radiation sterilization in drug susceptibility testing.

It was found that ¹H-NMR is a quite attractive method for quality control of plant metabolites as it allows the recognition of a broad metabolome, detecting diverse groups of compounds such as amino acids, carbohydrates, organic acids, phenolics, terpenoids and flavonoids. **Figure 1A** shows the ¹H-NMR spectrum of pure isolate (EBPCF₃) from bark of *Wrightia tomentosa*.

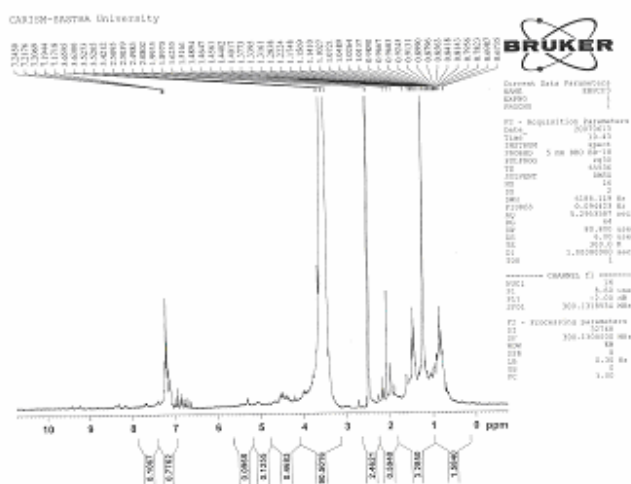


FIG. 1A: SLICE THROUGH THE GRAPH REPRESENTING 1H-NMR SPECTRUM OF ONE EXTRACT COMPONENT EBPCF3

Isoflavone moiety was identified in the ¹H-NMR spectra. Signals at $\delta=7.47$ (H-8; s), $\delta=7.43$ (H-6; s), $\delta=8.21$ (H -5; s), $\delta=7.68$ (H -7), $\delta=7.2176$ (m), $\delta=2.68$ (H -2; s), $\delta=7.38$ (H -5¹) and $\delta=7.74$ (H -4¹; s) were assigned to the protons of isoflavone moiety respectively.

NMR based plant analysis typically employs one dimensional ¹H-NMR methods to minimize sample acquisition times and hence maximize throughput. However, the spectral congestion of one-dimensional ¹H-NMR limits the number of metabolites, which can be uniquely identified and quantified. For overcoming this congestion, ¹H-¹H COSY techniques were applied to the analysis and definitely improved the resolution of the ¹H-NMR spectra. The characteristic signals identified from COSY spectra (**Figure 1B**) are at $\delta=7.7$ (d), $\delta=7.2$ (m), $\delta=6.6$ (q), $\delta=3.6$ (b s), $\delta=2.5$ (s), $\delta=2.1$ (t) and $\delta=1.2$ (t) for the presence of isoflavone.

The signal at $\delta 2.68$ was ascribed to the proton of oxygen bearing ring chromane function at C-2, characteristic for isoflavones. This was supported by the ¹³C-NMR where the C-2 ether carbon was observed at $\delta 86.6$ as well as by ¹H-¹H COSY experiments.

The ¹³C-NMR spectra (in DMSO-d₆) revealed 6 distinct carbon resonances. The signals of C-4 keto carbon of chromane ring and the substituted ring carbon of ketone at C-1¹ position were detected at $\delta 202.9$ and $\delta 210.6$. The characteristic positions of the aromatic hydrocarbon (C-7, $\delta 128$), cycloalkene carbon (C-3, $\delta 22.6$) and the ether

carbon (C-2, δ 86.6) were in accordance with the observed ^{13}C -NMR spectra (**Figure 2A**) for the presence of isoflavones. The strong resonance of δ 104.4 belong to the unsubstituted benzene carbons between C-1 and C-8 as well as with C-4 and C-5. H-2 and H-3 of chromane were identified at δ 2.68 and δ 3.13 as singlets, respectively from ^1H -NMR spectra. The assignments were confirmed by the HMBC spectra (**Figure 2B**) in which H-2 and H-3 correlated with C-2 at δ 86.6 and C-3 at δ 22.6. The clusters of ^1H -NMR proton ($\delta=3.5$) at H-2 and H-3 in HMBC spectra was very well correlated with the assignments of C-2 and C-3 as $\delta=70$ and $\delta=25$ in the HMBC spectra.

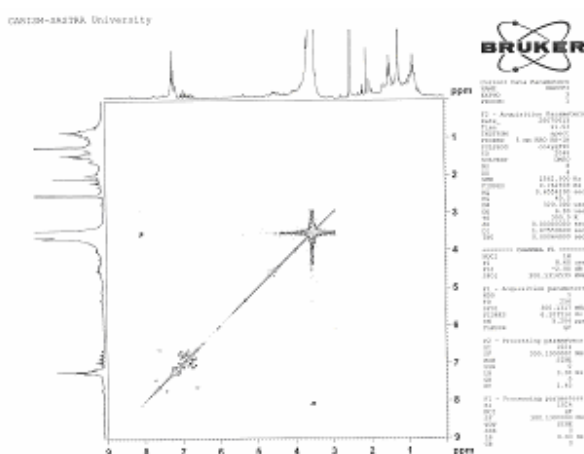


FIG. 1B: COSY SPECTRUM RECORDED WITH A PURE ISOLATE-EBPCF3 OF WRIGHTIA TOMENTOSA

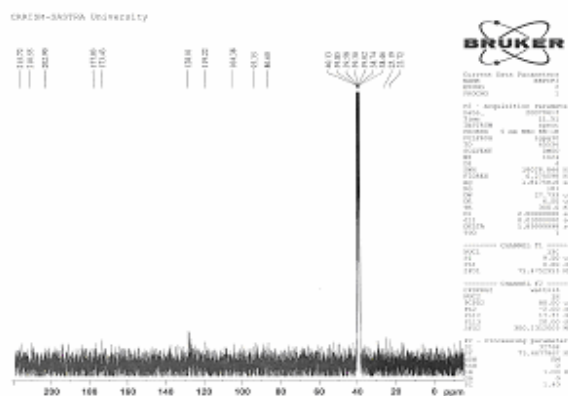


FIG. 2A: ^{13}C -NMR SPECTRUM OF AN ISOFLAVONE FROM A COLUMN PURIFIED COMPONENT EBPCF3 OF WRIGHTIA TOMENTOSA

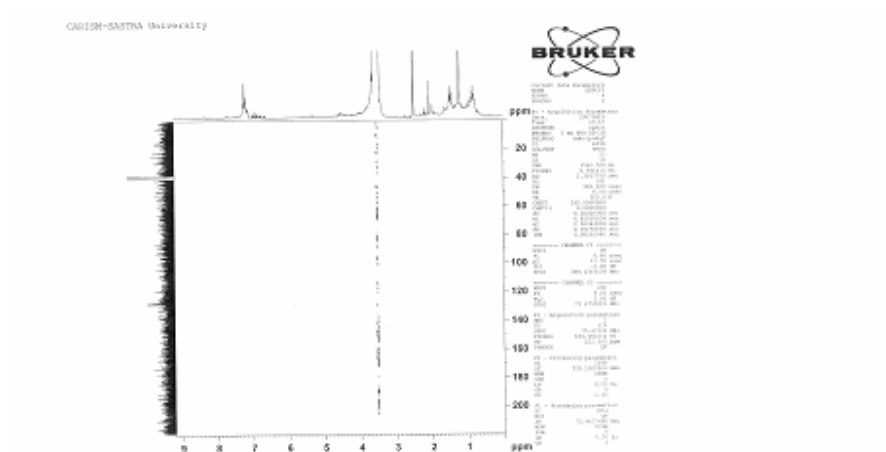
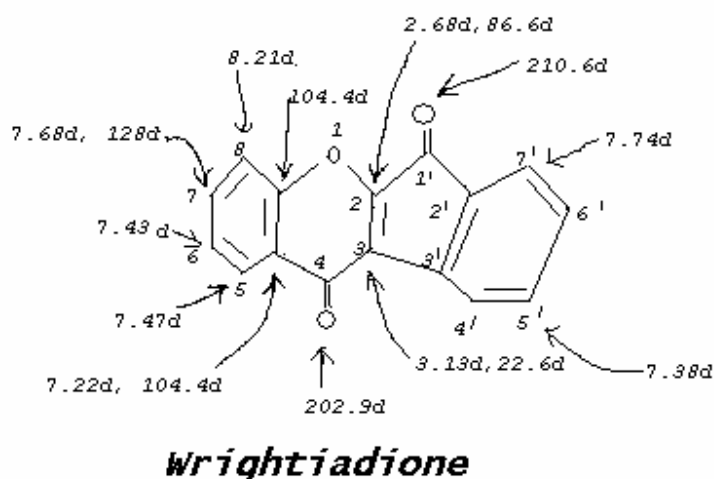


FIG.2B: HMQC SPECTRA ACQUIRED WITH WRIGHTIA TOMENTOSA BARK ISOLATE EBPCF₃



Hence the final structure of pure lead isolate (EBPCF₃) was elucidated as wrightiadione, a predominant isoflavone. Thus the test compound VII (EBPCF₃) exhibits maximum anti-mycobacterial potency due to the presence of isoflavone wrightiadione as predominant active constituent. Further investigation is needed to identify the mechanistic approach of isolated flavonoid component for anti-tubercular action.

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