IN-VITRO ANTIMYCOBACTERIAL EFFECTS OF ONE PURE ALKALOID LEAF ISOLATES OF *WRIGHITA TOMENTOSA*–AN EXPLORATORY INVESTIGATION

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Summary

In the traditional Indian medicine, the leaves of *Wrightia tomentosa* are administered for the relief of toothache. Some of the beneficial effects observed in patients suggest an antimycobacterial potency of *Wrightia tomentosa* isolates from alcoholic leaf extract. In this study, an alkaloidal pure component was isolated, identified and tested *in-vitro* against *Mycobacterium tuberculosis* H_{37} Rv strain using Versa TREK rapid culture system in need of emergence of multidrug resistance. The pure component from column (100% Ethyl acetate) was identified as alkaloid by preliminary chemical analysis (Dragendroff's reagent); TLC analysis (Rf: 0.63) and melting point analysis (85–87^{0}C). Further, the isolate was characterized by I.R. (2922–2852cm^{-1}; 1641cm^{-1}; 666cm^{-1}), ^{1}H NMR (1.222 δ Methylene proton; 1.6δ methyl proton; 3.5δ ring substituted methylene proton), ^{13}C NMR (150δ; 136δ; 126δ for the hetero aromatic compound) & Mass (M^+ 81) for the presence of N–Methyl pyrrole after elucidation. Leaf isolate N–methyl pyrrole showed time for positivity in 19.9 hours with respect to control (15.5 hours). Even though the isolated pure alkaloid withstand time for sustained growth as that of control (susceptible), N–methyl pyrrole are considered to be resistant by considering 3 days growth time period as per standard FDA protocol. These findings imply a potential application of the fraction as antimycobacterial agents and to be tested further clinically in-vivo.

Key words: *Wrightia tomentosa*, *Mycobacterium tuberculosis*, Versa TREK system, Isolate, Lead identification

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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 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 versa trek mycobottles with plant based isolates obtained from leaf 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(IC_{50} 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 leaf isolate of the same herbal *W.tomentosa.*

Materials and Methods

I. Plant material

The leaves of *Wrightia tomentosa* were collected from the hills of Yercaud forest, Salem district of Tamilnadu and identified **(16)**, authenticated by an acknowledged Botanist, Professor M.B.Viswanathan, Coordinator, Centre for Herbal Drug Discovery & Development of the Research Department of Bharathidasan University, Tiruchirapalli, Tamilnadu, India and the voucher specimen was deposited thereafter at Bharathidasan University(BDUT/ 545).

II. Extraction and Isolation

The leaves of *Wrightia tomentosa* were dried at room temperature and reduced to a coarse powder. The powdered material was subjected to qualitative tests **(17)** 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^{o}C. The extracts were concentrated & the solvent was completely removed by Rotary vacuum evaporator (Buchi).

The concentrated ethanol leaf extract (3 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) 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.

A column of 5.0 ft. length and 16 mm of internal diameter was 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 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 **(18)**. 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: 0.63 (Toluene: chloroform: ethanol: 28.5: 57: 14.5) for alkaloid was designated as $ELPCF_3$. Hence one pure isolated alkaloid component ($ELPCF_3$) from leaf in a single dose was selected for drug susceptibility testing against typical *Mycobacterium tuberculosis* H37 Rv using Versa Trek rapid culture system.

III. Methodology

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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 **(19)** has combined a liquid culture medium (versa trek myco broth), a growth supplement (versa trek myco GS) and a specific concentration of ethanolic leaf pure isolate 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 isolates of *Wrightia tomentosa* was carried out at Doctor's Diagnostic Center R & D Labs, Tiruchirappalli, Tamilnadu, India. A single dose of pure alkaloid leaf isolate (60 mg) was taken and designated as $ELPCF_3$-60. They were evaluated *invitro* against *M.tuberculosis* H37 Rv (ATCC 27294) using Middlebrook 7H9 broth as the nutrient medium containing ADC growth supplement **(20,21)**.

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 compound ELPCFG-60 in a single dose (60mg from leaf) was 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 **(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. 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).

Fig. 1 Instrument for antimycobacterial drug susceptibility testing by Versa TREK rapid culture system

Results

The results of the test compound I of typical mycobacterial susceptibility testing were shown in Table (1).

Table (1)

TYPICAL MYCOBACTERIAL SUSCEPTIBILITY TEST FOR *WRIGHTIA TOMENTOSA* ETHANOLIC ALKALOID PURE ISOLATE

S.No	Type of sample	Sample access number	Concen--tration of the sample / 4200µl DMSO	Drug loaded (µl)	Days /Hours of Identifiable Growth (Time for positivity)	Status
1	Ethanol leaf Pure isolate-3 (Test compd.I)	ELPCF G-60	60 mg	500	Growth identified in 19.9 hours	Resistant
2	Leaf control	LEAFC	Drug free medium	500 (Sterile distilled water)	Growth identified in 15.5 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 compound I showed growth in 19.9 hours whereas the leaf control shown remarkable growth in 15.5 hours

respectively. Even though the isolated test compound (I) withstand time for sustained growth as compared with control and found to be susceptible, the test compound of leaf isolate(I) 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. The test compound time for positivity was clearly evidenced with the following figures 2 & 3 attached herewith.

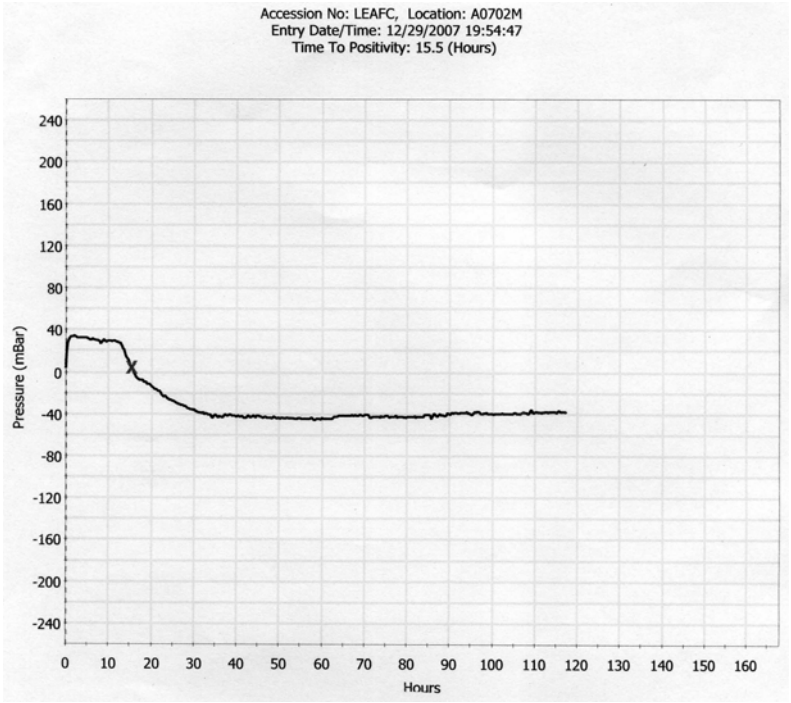

Fig. 2 Control graph of *Wrightia tomentosa* leaf extract against *M. tuberculosis*

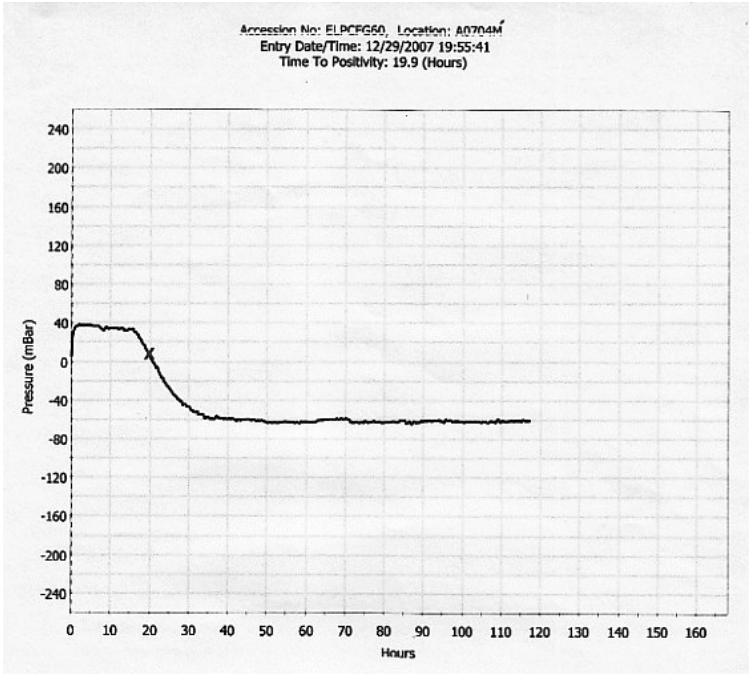

Fig. 3 Time positivity graph of *Wrightia tomentosa* leaf isolate $ELPCF_3$-60 **against *M. tuberculosis***

Structure elucidation of pure alkaloid leaf isolate ($ELPCF_3$-60):

Isolated pure component ($ELPCF_3$) from running flash column (100% ethyl acetate) was found to be susceptible against typical *Mycobacterium tuberculosis* without considering the standard FDA protocol duration. Hence, its final structure was confirmed by Melting point, ^{1}H-NMR, ^{13}C-NMR and Mass spectral datas. Preliminary phytochemical screening and TLC results have revealed that the pure component ($ELPCF_3$) was basically an alkaloid class of compound.

General experimental procedure: The melting point was recorded on Mettler-FP-80/82 hot stage apparatus. IR spectra was recorded on FT-IR (Perkin Elmer) spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were measured using a Bruker DRX 300 instrument with DMSO-d_6 as solvent (300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C). EIMS measurements were carried out with a Quadrupole instrument in positive ionization mode (Finnigan MAT 44s, San Jose, CA, USA) at 70 eV.

Results: Melting point of the isolated fraction exists in the range of 85-87^{0}c.IR spectra shows characteristic functional group bands for the presence of N-methyl pyrrole at 2922-2852 cm^{-1}, 1641 cm^{-1} and 666 cm^{-1}.^{1}H-NMR spectra reveals the presence of methylene proton of N-methyl pyrrole at 1.222 δ; methyl proton at 1.6 δ and a methylene proton as ring substituent at 3.5 δ. This was further confirmed with ^{13}C-NMR at 150 δ; 136 δ & 126 δ indicating the structure of hetero aromatic compound. Mass spectral analysis clearly indicates the evidence of N-Methyl pyrrole as the final structure of the isolated alkaloid pure component with the corresponding molecular ion peak at 81(M^+). All these spectral data suggests that the alkaloid leaf isolate ($ELPCF_3$) eluted from column was found to be N-Methyl pyrrole.

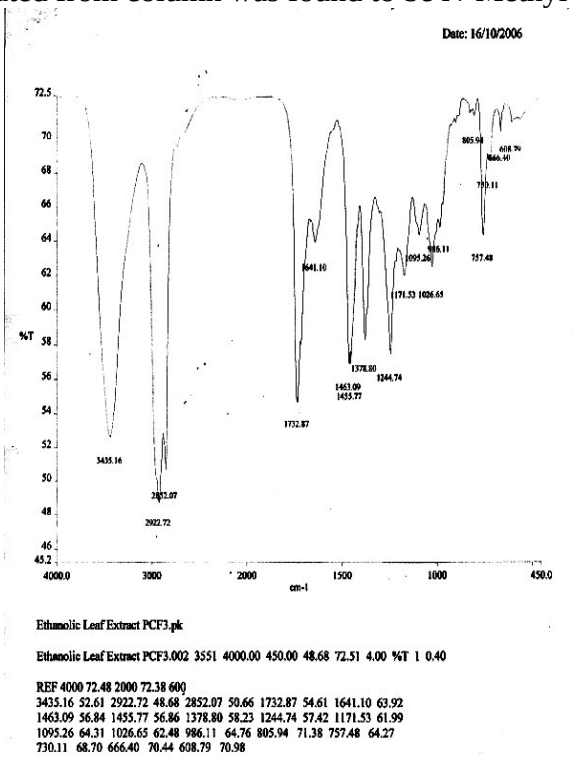

Fig. 4 Slice through the graph representing IR spectra of one extract pure alkaloid component $ELPCF_3$ from *Wrightia tomentosa*

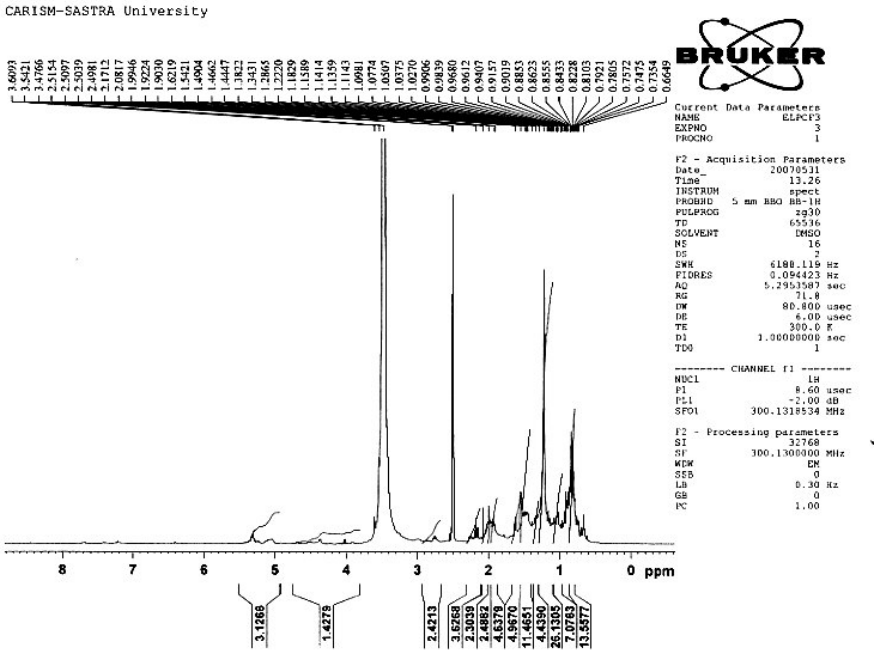

Fig. 5 ^{1}H-NMR spectra of pure alkaloid ELPCF$_3$ from *Wrightia tomentosa*

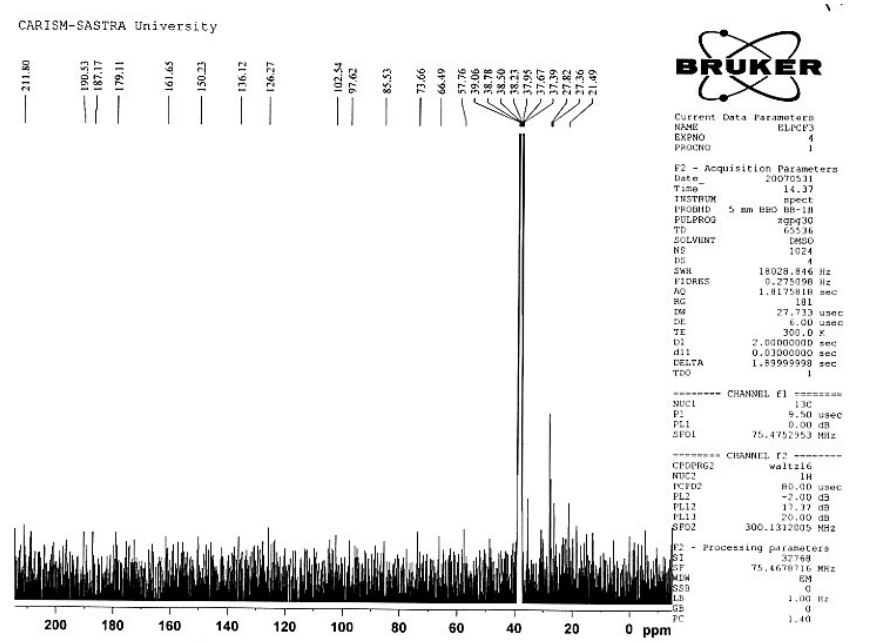

Fig. 6 ^{13}C-NMR spectrum of an alkaloid from a column purified component ELPCF$_3$ of *Wrightia tomentosa*

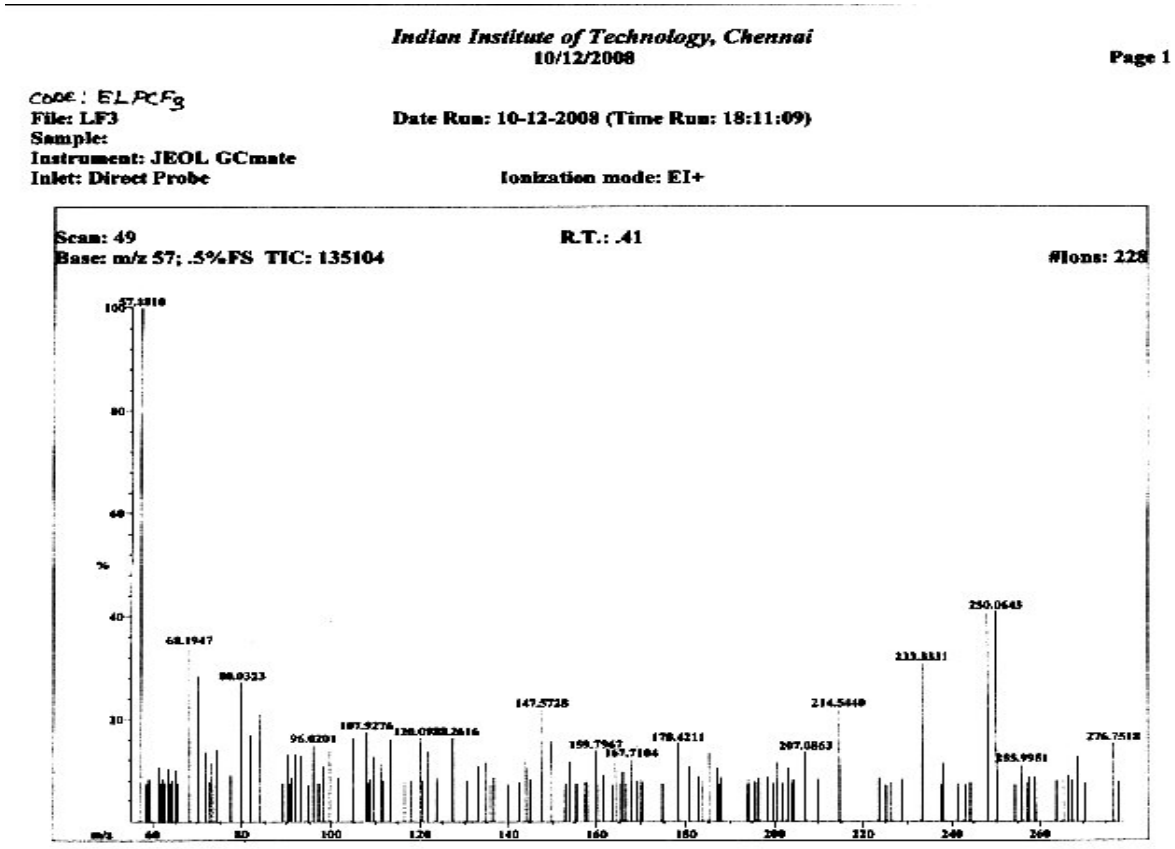

Fig. 7 Mass spectrum of an alkaloid component ELPCF$_3$ of *Wrightia tomentosa*

Discussion

Worldwide, tuberculosis remains the most frequent and important infectious disease causing morbidity and death. The World Health Organisation estimates that about eight to ten million new TB cases occur annually worldwide and the incidence of TB is currently increasing. These situations, particularly the global resurgence of TB and the rapid emergence of MDR-TB, underscore the importance of the development of new antituberculous drugs **(24).**

Immunoadjunctive therapy appears to be promising with the development of new classes of immunomodulators other than cytokines (IL-2, IFN-gamma, GM-CSF, IL-12, etc.) belonging to the category of ATP and its analogues, which potentiate macrophage antimycobacterial activity via purinergic P2 receptors **(24).** Certain novel oxazo-imidazole derivative drugs like OPC-67683 have the potential to shorten the total duration of treatment, provide improved efficacy against MDR-TB, be useful in treating HIV co-infected patients, and target latent TB infections with a distinctive characteristic as a subclass mycolic acid inhibitor **(25).**

The relevance of apoptosis to kill intacellular *Mycobacterium tuberculosis* was studied with phorbol differentiated THP-1 cells. Microparticles containing isoniazid and rifabutin induced intrinsic apoptosis and bacterial killing equivalent to that with dissolved drugs and maximally enhanced purinergic P2 receptor activity, while drug free microparticles induced apoptosis via a different mechanism without killing bacteria **(26).**

The chemokine receptor CXCR3 plays a significant role in regulating the migration of Th 1 cells. CXCR3 plays a role in modulating the cellular composition of teberculous granuloma; CXCR3 impairs antimycobacterial activity in chronic tuberculosis and in the

absence of CXCR3, mice exhibit a heightened state of CD4+ T lymphocyte activation in the chronic phase of infection that is associated with enhanced CD4+ T cell priming. Hence, CXCR3 can attenuate the host immune response to *M.tuberculosis* by adversely affecting T cell priming **(27).** Since our isolated test compound ELPCF3, N-Methyl pyrrole possess the basic nitrogen containing skeleton which is similar to that of the ATP analogue, it is presumed that the N-Methyl pyrrole potentiate macrophage antimycobacterial activity via purinergic P2 receptors.

Acknowledgement

The authors are very much thankful to Mr. Abhinav Agarwaal, Secretary General, IIMT Group of Colleges, Meerut, India for giving us the constant encouragement and untiring moral support in carrying out this research project in successful manner. Also, We remain thankful to Dr. P. K. Rath and Mr. Suresh of Doctors Diagnostic Center, Trichy, Tamilnadu, India for their valuable technical suggestions.

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