Research Article

Evaluation of Anti-Tuberculosis Potential and GC-MS Analysis of Selected Medicinal Plants of Rutaceae

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ABSTRACT

Background/Objectives: Tuberculosis (TB) is one of the oldest serious infectious diseases known to affect human beings and shows high rate of mortality worldwide. Increasing incidences of MDR and XDR-TB highlight the necessity of new anti-tuberculosis medications. Chemotherapeutic agents cause adverse side effects and other serious health problems. Nowadays, bioactive compounds derived from plants are broadly recognized for their anti-tuberculosis effects.

Methods: The present study was designed to investigate the antimycobacterial potential of methanolic extracts of leaf of plants belonging to the family Rutaceae- *Limonia acidissima* and *Murraya koenigii*. BACTEC Micro MGIT system was used for the analysis of antituberculosis activity. Gas Chromatography- Mass Spectrometry (GC-MS) technique was used to identify active compounds present in selected plants.

Results: The assay results clearly demonstrate that selected clinical isolate of MTB were inhibited by leaf extract of both the plants at 500µg/ml concentration; particularly methanolic leaf extract of *M. koenigii* is more efficient than *L. acidissima*. Further, GC-MS analysis of methanolic leaf extract showed the occurrence of 31 major compounds in *M. koenigii* and 27 major compounds in *L. acidissima*. These compounds belong to group of phytochemicals having proven therapeutic potential.

Conclusion: Taken together, the selected plants extracts may be used for the development of novel herbal medicines against mycobacterial diseases. Simultaneously, future studies require isolation and detailed characterization of the lead compounds and their evaluation for anti-TB activity.

Keywords: Tuberculosis, Rutaceae, MDR, GC-MS, Anti-mycobacterial activity.

INTRODUCTION

Tuberculosis is mainly caused by *Mycobacterium tuberculosis*[1], belonging to the genus *Mycobacterium*. Other causal strains of *Mycobacteria* are *M. africanum, M. bovis, M. microti, M. leprae and M. avium*. It transmits mainly through air, when infected person cough, sneeze, or transmit their saliva. The commonly observed symptoms of TB are weakness, chronic cough, fever, weight loss, night sweats and chest pain.

According to the WHO Global Tuberculosis Report 2023, new cases of tuberculosis reported in 2022 were 10.6 million. Globally in 2022, 1.3 million deaths were caused by TB, thus, making this infectious disease life threatening [2].

Commonly used drugs in the treatment are isoniazid, rifampicin, ethambutol, pyrazinamide etc [3]. These drugs have adverse side effects and *Mycobacteria* can gain resistance against them easily. Disease control is difficult due to the appearance of Multi-drug Resistant (MDR)) and Extensively- drug Resistant (XDR) strains. MDR is caused by the strains that are resistant to the action of at least isoniazid (INH) and rifampicin (RMP), drugs used in first line therapy [4,5]. The XDR is referred as a strains type that are resistant to drugs used in first line therapy along with any fluoroquinolone and at least one of the three-capreomycin, kanamycin, and amikacin, injectable drugs used in second line therapy [6,7]. Moreover, the treatment is more expensive in case of MDR and XDR as compared to drug-susceptible strains [8].

Due to genetic mutation and adaptability to varying environmental conditions, the infectious microorganisms have improved their infectivity and viability [9]. This warrants the novel drug discovery and development with improved efficacy against evolving strains [10]. Herbal products have unequivocal potential for developing alternative medicines with least side effects and with limited or no toxicity [11]. Medicinal plants contain bioactive molecules with various functional groups which are attributed to multiple mechanisms to combat microbial infections; hence the occurrence of resistance against phytochemicals is comparatively less [12].

Furthermore, plant secondary metabolites can disrupt the mycobacterial membrane structure, interfere with DNA replication, translation and gene regulation, coagulation of cytoplasm contents, interfere with metabolic processes and inhibit enzymes required for cell wall synthesis [13]. Thereby, it can be stated that phytomedicine based techniques can be used in reducing the incidence of tuberculosis along with MDR and XDR strains [14].

Limonia acidissima, commonly known as Kaith is a natural plant used in traditional system of medicine for the treatment of various diseases. Leaves are astringent, hepatoprotective, carminative. The bark and leaves are used in digestion disorders [15]. The bark is useful in liver diseases. Fruits of *Limonia* are reported as stomachic, stimulant, astringent, diuretic, cardiotonic, used in the treatment of cough, hiccup, tumours, opthalmia and leucorrhoea etc. [16].

Murraya koenigii (Curry leaf tree) is used for flavoring soups, curries and other food preparations. It is used as a stimulant, antidysentric and also effective against diabetes mellitus. Leaves are used as stomachic, purgative, febrifuge, antianemic and also checking vomiting [17].

In the present study these two plants belonging to family Rutaceae have been investigated with an objective to assess antimycobacterial activity of methanolic extracts of leaves by using BACTEC™ Micro MGIT™ assay. GC-MS analysis has also been performed for profiling of bioactive compounds of these selected medicinal plants.

MATERIALS AND METHODS

Collection and preparation of plant material

Leaves of *Murraya koenigii* were collected from Department of Botany, University of Rajasthan Jaipur and leaves of *Limonia acidissima* were collected from Tonk district (Rajasthan). Identification of the plant material was carried out in the herbarium, Department of Botany, University of Rajasthan, Jaipur. Voucher specimen of both the selected plant species (*L. acidissima*; RUBL 211890 and *M. koenigii*; RUBL 211431) have been deposited in the herbarium of the Department. The leaves of plants were washed and dried under shade. The dried material was mechanically powdered and stored in an airtight container.

Preparation of extracts

Methanolic leaf extracts of *L. acidissima* and *M. koenigii* were used for investigation of the efficacy against *M. tuberculosis* using the BACTEC Micro MGIT system. For this, the Sonication (ultrasonic) technique was used for the extraction of plant material. The dried leaf powder (5gm) was mixed in 50ml of methanol and sonicated for 15 minutes. The processed plant extract was centrifuged and filtered using Whatman filter paper No.1. The sample extract was air-dried. The dried extract of each plant was dissolved in dimethyl sulfoxide (DMSO) (0.1%) to achieve the final concentration of 500µg/ml. Anti- TB activity was done at Magnum Diagnostics and Research Centre, Jaipur, India.

Assessment of antimycobacterial activity

BBL™ MGIT™ tubes (7ml 7H9 middle brook broth) were labeled. Isoniazid was used as positive control or standard. DMSO as negative control/ growth control which is without any drug. Methanolic leaf extract of both the plants are used as samples. MGIT growth supplement (0.8ml) was added aseptically to each MGIT tube. Then 0.5ml of growth suspension was added. Thereafter, 0.1ml of the standard, growth control and plant sample was added to the respective BBL™ MGIT™ tubes followed by the introduction of 0.5ml of *Mycobacterium* suspension. The protocol was handled under Class III bio safety cabinet. Then the tubes were incubated at 37 °C for 15 days and detected by MGIT UV detector in every 24 hours to examine growth unit in each tube and confirmed by ZN (Ziehl-Neelsen) staining technique.

GC- MS analysis

The Gas chromatography-Mass spectrometry (GC-MS) analysis of methanolic leaf extracts of selected plants was conducted at Advanced Instrumentation Research Facility (AIRF), Jawahar Lal Nehru University, New Delhi. It is a combined analytical technique, used for screening and identification of different compounds present in plants.

RESULTS AND DISCUSSION

The results of inhibitory effect of methanol extract of leaf of *L. acidissima* and *M. koenigii* showed that selected clinical isolate of MTB was susceptible to the leaf extract of both the plants at 500µg/ml concentration.

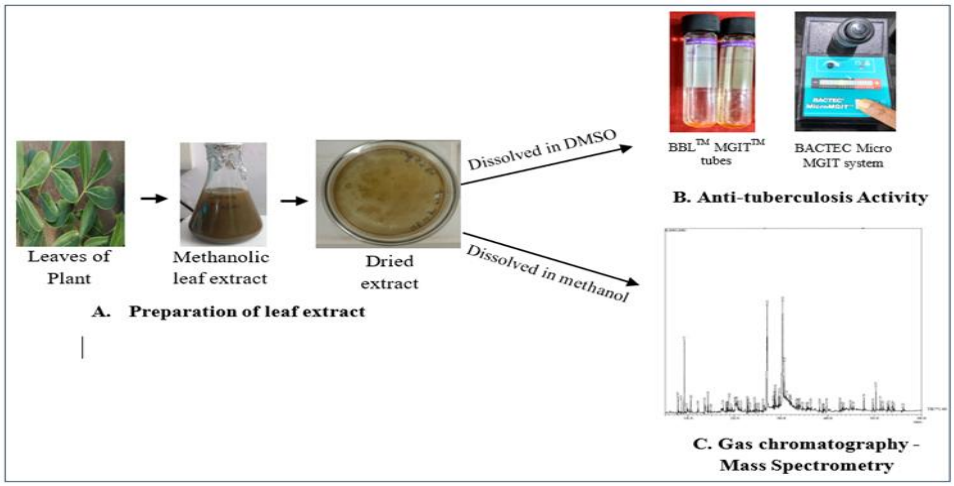

Figure 1: A summary of approach used for study. A. Preparation of methanolic leaf extract of selected plants, B. Anti-tuberculosis activity using BACTEC Micro MGIT system. C. GC- MS analysis of methanolic extract.

BBL™ MGIT™ tubes are used in BACTEC™ Micro MGIT™ assay. The BBL (from Becton Dickinson) MGIT (Mycobacteria Growth Indicator Tube) contains 7 mL of Middlebrook 7H9 medium which supports the growth of mycobacteria, a fluorescent compound Tris 4, 7 - diphenyl-1, 10-phenanthroline ruthenium chloride pentahydrate embedded in silicone at the bottom. This compound is sensitive to the oxygen. Initially, a large amount of dissolved oxygen in broth quenches emissions from the compound, hence low fluorescence is detected. Later, dissolved oxygen is consumed by microorganisms which are actively respiring and high fluorescence is observed at 365 nm in UV illuminations which are sensed by the BACTEC's sensor [18,19]. Growth can also be observed by turbidity or flakes in the culture medium.

Results of growth were observed in MGIT UV detector (works on fluorescence-based principle) in every 24 hrs for 15 days. BBL™ MGIT™ tube containing methanolic extract of leaf of *M. koenigii* as a drug (500 µg/ml) showed 7 GU at day 15 and least fluorescence was detected while leaf of *L. acidissima* (500 µg/ml) exhibited 9 GU. In case of standard drug Isoniazid (INH) (0.1 µg/ml) it is observed as 10 GU and in growth control tube or tube without any drug (0.1 µg/ml) as 20 GU and maximum growth of Mycobacteria and fluorescence was observed.

In BACTEC™ Micro MGIT™ assay, generally more than growth control (GC) tube is considered as positive growth while less than GC is negative growth or inhibited growth [20]. In present study, DMSO containing negative control tube or GC tube showed 20 GU while methanolic extracts of leaf of both the selected plants and Standard drug showed significant growth inhibition (Table-1). Although Standard drug shows better result at lower concentration than crude plant extracts due to the presence of mixture of various bioactive compounds [21]. This was further confirmed by ZN staining technique which showed absence of Mycobacterium in standard and leaf extracts of both the selected plants. Results from present study showed that methanolic leaf extract of both *L. acidissima* and *M. koenigii* may be used as anti-tubercular drug. Although, methanolic leaf extract of *M. koenigii* (GU- 7) exhibited more efficiency as compared to *L. acidissima* (GU- 9).

The present analysis assumed that the isolate was considered as susceptible by the methanolic leaf extracts of selected plants which may be attributed to presence of various bioactive compounds. Moreover, data analysis of our study significantly revealed that BACTEC™ Micro MGIT™ is an efficient, fast and accurate assay to find out anti-TB activity in plant extracts within 13-15 days.

Methanolic extract of leaf showed anti- tuberculosis activity hence GC-MS analysis was done for presence of various compounds. The GC-MS study of leaf of *L. acidissima* exhibited the occurrence of 27 major compounds (Table- 2; Fig- 2) and leaf of *M. koenigii* 33 major compounds (Table- 3; Fig- 3). Compounds similar to flavonoids, alkaloids, sterols, coumarins, terpenoids, fatty acids and derivatives have been identified that could contribute the medicinal quality of the selected plants.

Results from the anti-tuberculosis assay (Table 1) revealed that the susceptibility recorded to the methanolic leaf extract was considered remarkable. This finding justified the presence of bioactive compounds in both the selected plant species of Rutaceae which are responsible for the anti-TB activity. Also, a previous study by Patil et al [22] had showed a significant antimycobacterial activity of leaves of *Murraya koengii* by Micro-plate Alamar blue assay (MABA). Furthermore, earlier studies on extracts from some members of Rutaceae family- *Aegle marmelos, Zanthoxylum leprieurii*, showed antimycobacterial activity [23,24].

Table 1: Anti-tuberculosis activity (GU) of methanolic leaf extract of *L. acidissima* and *M. koenigii.*

Sample	Conc. (µg/ml)	Day 1 to Day 15															Interpretation
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
		Growth Unit															
L. acidissima	**500**	6	6	6	6	6	7	7	7	7	7	8	8	8	9	**9**	No growth or growth inhibited
M. koenigii	**500**	6	6	6	6	6	6	6	6	7	7	7	7	7	7	**7**	No growth or growth inhibited
Std Drug (INH)	**0.1**	6	6	8	8	8	8	8	9	9	9	9	9	10	10	**10**	No growth or growth inhibited
Growth Control (DMSO)	**0.1**	6	6	8	8	8	9	9	10	11	13	16	16	18	19	**20**	Growth Positive

More than or equal to GC- Growth positive; Less than GC- No growth or growth inhibited.

Table 2: Phytoconstituents present in the methanolic leaf extract of *L. acidissima* as revealed by GC-MS analysis

S. N	R.Time	Name of compound	Molecular Formula	Molecular Weight	Peak Area (%)	Biological activity
1.	7.807	2,3-Dihydro-3,5-Dihydroxy-6-Methyl-4H-Pyran-4-one	$C_6H_8O_4$	144	1.53	Strong antioxidant and neuroprotective *activities*
2.	9.172	Benzene, 1-Methoxy-4-(2-Propenyl)-Estragole	$C_{10}H_{12}O$	148	5.05	Direct inhibition of Na^+channels
3.	9.725	2,3-dihydro-benzofuran	C_8H_8O	120	0.79	Anticancer
4.	10.605	Benzeneacetic acid	$C_8H_8O_2$	136	2.07	Trypsin inhibition
5.	12.061	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150	0.71	Antioxidant Antimicrobial Anti-inflammatory
6.	13.546	4-Hydroxybenzaldehyde	$C_7H_6O_2$	122	1.19	Antimicrobial
7.	14.290	Benzene, 1,2-dimethoxy-4-(2-propenyl)- Methyl eugenol	$C_{11}H_{14}O_2$	178	0.96	Anticonvulsant and anesthetic
8.	22.762	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	0.85	Larvicidal and repellent activity
9.	22.906	2(4H)-Benzofuranone, 5,6,7,7A-tetrahydro-6-hydroxy	$C_{11}H_{16}O_3$	196	0.70	Antimicrobial, anti-inflammatory
10.	24.329	2,6,10-Trimethyl,14-ethylene-14-Pentadecane	$C_{20}H_{38}$	278	0.56	Antiproliferative
11.	27.025	Pentadecanoic acid	$C_{15}H_{30}O_2$	242	20.30	Antimicrobial
12.	28.581	7H-Furo[3,2-g][1]benzopyran-7-one, 4-methoxy	$C_{12}H_8O_4$	216	1.14	Cytotoxic, antitumoral, and antimalarial activities
13.	28.766	Heptadecanoic acid	$C_{17}H_{34}O_2$	270	1.36	Antimicrobial
14.	30.338	6-Octadecenoic acid, (Z)	$C_{18}H_{34}O_2$	282	28.23	Antimicrobial
15.	30.667	Octadecanoic acid	$C_{18}H_{36}O_2$	284	3.11	Cancer preventive, Insectifuge
16.	33.284	Bicyclo[3.1.1]hept-2-ene-2-ethanol, 6,6-dimethyl-	$C_{11}H_{18}O$	166	0.66	Antimicrobial activity
17.	33.620	9-Octadecenoic acid (Z)-	$C_{18}H_{34}O_2$	282	0.90	Anticancer
18.	36.360	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	330	0.56	Hemolytic, antioxidant, pesticide
19.	38.294	2H-1-Benzopyran-2-one, 7-[(3,7-dimethyl-2,6-octadienyl)oxy]-, (E)-	$C_{19}H_{22}O_3$	298	0.69	Anticancer activity
20.	39.796	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester	$C_{21}H_{40}O_4$	356	2.12	Antibacterial activity
21.	42.643	Squalene	$C_{30}H_{50}$	410	0.73	Chemo-preventive activity
22.	47.784	dl-.alpha.-Tocopherol	$C_{29}H_{50}O_2$	430	1.23	antimicrobial, Anti-inflammatory, antioxidant
23.	49.721	Ergost-5-en-3-ol, (3.beta.)	$C_{28}H_{48}O$	400	1.24	Antifungal
24.	50.375	Stigmasterol	$C_{29}H_{48}O$	412	3.61	Thyroid inhibitory, antiperoxidative and hypoglycemic effects.
25.	51.357	Cholest-5-en-3-ol, 4, 4-dimethyl-, (3.beta.)	$C_{29}H_{50}O$	414	1.50	Anti-inflammatory activity
26.	51.907	Stigmast-5-en-3-ol, (3.beta.)-	$C_{29}H_{50}O$	414	0.73	Anti-diabetic
27.	53.950	Lanosterol	$C_{30}H_{50}O$	426	1.48	Antifungal

Table 3: Phytoconstituents present in the methanolic leaf extract of *M. koenigii* as revealed by GC-MS analysis

S.N	R.Time	Name of compound	Molecular Formula	Molecular Weight	Peak Area (%)	Biological activity
1.	4.183	Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-	$C_{10}H_{16}$	136	1.45	Antimicrobial activity
2.	6.811	1-Octyn-3-ol	$C_8H_{14}O$	126	0.53	Antibacterial activity
3.	7.790	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	$C_6H_8O_4$	144	0.51	Antimicrobial, anti-inflammatory, antiproliferative
4.	9.327	sec-Butyl nitrite	$C_4H_9NO_2$	103	0.64	No activity reported
5.	9.847	2-furanmethanol	$C_5H_6O_2$	98	0.86	No activity reported
6.	14.098	2,4-diisopropenyl-1-methyl-1-vinylcyclohexane	$C_{15}H_{24}$	204	0.54	Antifungal activity
7.	14.890	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene- caryophyllene	$C_{15}H_{24}$	204	3.79	Anti-inflammatory, antinociceptive,neuroprotective, anxiolytic and antidepressant
8.	15.770	1,4,8-cycloundecatriene, 2,6,6,9-tetramethyl-, (E,E,E)- Humulene	$C_{15}H_{24}$	204	0.77	Anticancer
9.	16.760	alpha.-selinene	$C_{15}H_{24}$	204	0.55	Antioxidant and anti inflammatory
10.	17.360	beta.-D-Glucopyranose, 1,6-anhydro-	$C_6H_{10}O_5$	162	0.72	Activity not reported
11.	24.328	2,6,10-trimethyl,14-ethylene-14-pentadecne	$C_{20}H_{38}$	278	0.70	Antiproliferative
12.	24.746	1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester	$C_{16}H_{22}O_4$	278	1.28	Antifungal activity
13.	26.644	1,2-benzenedicarboxylic acid, bis(2-methoxyethyl) ester	$C_{16}H_{22}O_4$	278	0.84	Antifungal activity
14.	26.882	Pentadecanoic acid	$C_{15}H_{30}O_2$	242	5.23	Antifungal activity
15.	29.418	6-Octadecenoic acid, methyl ester, (Z)-	$C_{19}H_{36}O_2$	296	0.89	Antimicrobial activity
16.	29.611	2-hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-	$C_{20}H_{40}O$	296	4.95	Protect gastric mucosa
17.	30.047	D5-dodecene-1-ol	$C_{12}H_{24}O$	184	0.81	Flavor enhancing food additive
18.	30.289	6-Octadecenoic acid, (Z)-	$C_{18}H_{34}O_2$	282	16.45	Antimicrobial activity
19.	30.619	Octadecanoic acid	$C_{18}H_{36}O_2$	284	1.61	Antimicrobial activity
20.	31.376	Heneicosyl acetate	$C_{23}H_{46}O_2$	354	0.63	Antioxidant *activities*
21.	39.779	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester	$C_{18}H_{34}O$	266	2.46	Anticancer
22.	41.865	Pyrano[3,2-A]carbazole, 3,11-dihydro-10-methoxy-3,3,8-trimethyl- Koenimbine	$C_{19}H_{19}NO_2$	293	3.86	Antioxidant and protective *activity*
23.	43.124	Indolo[2,3-B][1,4]benzodiazepin-12(5H)-one, 10B,11-dihydro-10B-	$C_{16}H_{13}N_3O_2$	279	1.09	New Compound
24.	43.287	2,3,9,10-Tetrahydro-1,8-dioxa-7,12-diazadicyclopenta(b,j)phenanthrene	$C_{16}H_{12}N_2O_2$	264	0.86	No activity reported
25.	45.047	5-Methoxy-2-(p-methoxyphenyl)-8H-thieno(2,3-b)indole	$C_{18}H_{15}NO_2S$	309	5.56	Anticancer activity
26.	45.653	6,9,10-Trimethoxy-12H-benz(6,7)oxepino(2,3,4-i,j)isoquinoline	$C_{19}H_{17}NO_4$	323	20.65	Anticancer activity
27.	46.630	Pyrazine, tetrakis(1-methylethyl)-	$C_{16}H_{28}N_2$	248	1.29	Antifungal
28.	47.806	Vitamin E	$C_{29}H_{50}O_2$	430	4.04	Antidermatitic, Antileukemic, Antitumor, Anticancer, Hepatoprotective
29.	49.712	Ergost-5-en-3-ol, (3.beta.,24R)-	$C_{28}H_{48}O$	400	0.74	Anti-inflammatory effects
30.	51.930	Stigmast-5-en-3-ol, (3.beta.)-	$C_{29}H_{50}O$	414	2.72	Anti-diabetic
31.	56.134	dl-.alpha.-Tocopherol	$C_{29}H_{50}O_2$	430	0.97	Anti-inflammatory, antioxidant, antimicrobial

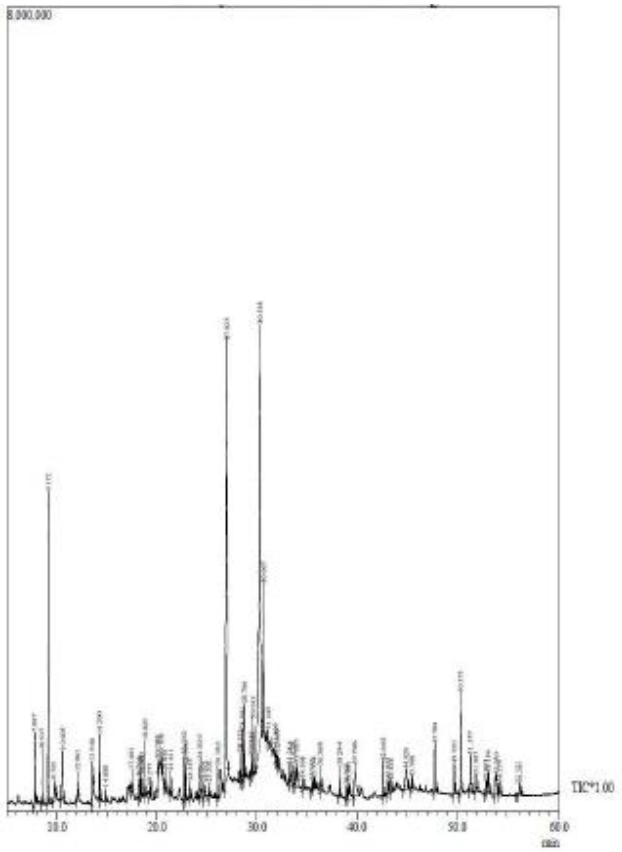

Figure 2: GC-MS chromatogram of methanol extract of leaf of *L. acidissima*

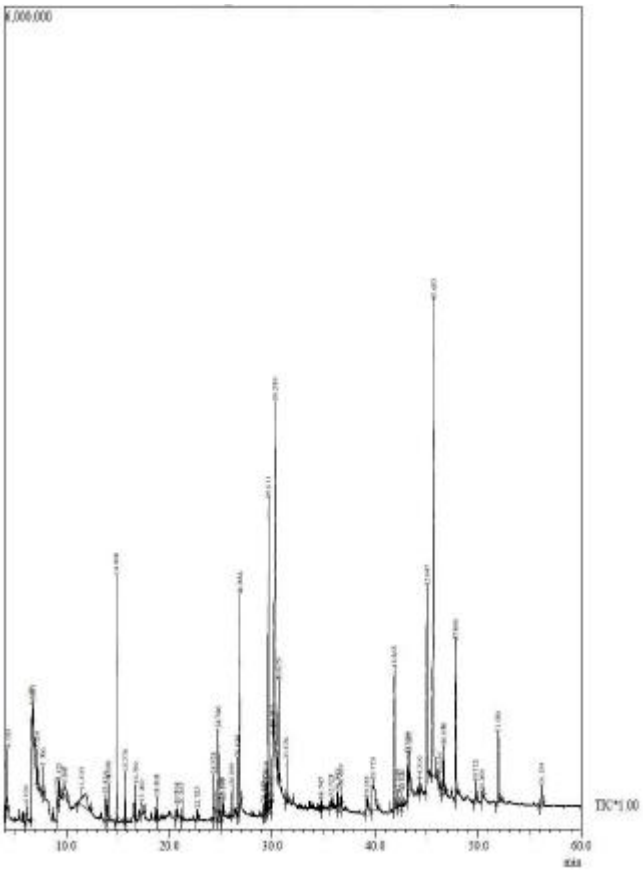

Figure 3: GC-MS chromatogram of methanol extract of leaf of *M. koenigii*

CONCLUSION

TB is one of the contagious diseases with high occurrence and dreaded global mortality rate. The increase of co-infections like HIV and the speedy appearance of antibiotic-resistant strains recommend an urgent need to develop effective and safe drugs with less or no side effect for eradication of TB.

The results of anti-tuberculosis activity by BACTEC™ Micro MGIT™ assay suggested that the selected plants of Rutaceae may have therapeutic value in the treatment of TB. Our GC-MS analysis showed a plentiful of chemical constituents in the leaf extracts of selected plants. However, further efforts are needed to isolate and investigate particular compound responsible for eliciting the anti-tuberculosis activity. Obviously, identification of particular compounds having anti-TB activity will accelerate the process of development of novel herbal medicines for the treatment of TB. Further, different algorithms based computational approaches could play an important role in screening plant active compounds, find out potential lead compounds against disease virulence of tuberculosis and will also help in drug designing.

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