Research Article

Isolation, Characterization of Bioactive Compounds and Evaluation of Anti-Tubercular Activity of *Ricinus communis* Linn.

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ABSTRACT

Tuberculosis is among the most serious infectious cause of global morbidity and mortality. Emergence MDR-TB is posing an increased threat to TB control programs and HIV fuels the TB epidemic. This led to stimulation in the research for the development of new drugs from plant origin showing anti-tubercular activity. *Ricinus communis* Linn. Commonly called as castor (family Euphorbiaceae) reported that it is traditionally used in the treatment of tuberculosis. No scientific evaluation has been conducted to check anti-tubercular activity of *Ricinus communis* Linn. This has inspired us to evaluate the anti-tubercular activity scientifically and identify the phytoconstituents responsible for the same from the plant. In present study, *Ricinus communis* Linn. leaves were selected for the pharmacological evaluation for possible anti-tubercular activity, further isolation and characterization of bioactive compounds from ethanol extract. Total 5 compounds were isolated from ethanol extract and evaluated for In -vitro antitubercular activity against *Mycobacterium tuberculosis* H37Rv strain by NRA (Nitrate reductase assay) method. Most of the tested compounds showed considerable antitubercular activity compared to standard antitubercular drugs like rifampicin and isoniazid. HPTLC, IR, ^{1}HNMR and GC-MS study of isolated two compounds CF-1 and CF-2 gave satisfactory results for confirmation of the structure as oleic and linoleic acid with significant antitubercular activity. Oleic and linoleic acid with significant anti-TB potential and the most active compounds could be useful as a template for treatment of tuberculosis in future.

Keywords: *Mycobacterium tuberculosis*; Castor, NRA, oleic acid; Linolic acid.

INTRODUCTION

Tuberculosis (TB) is a disease caused by the infection with members of *Mycobacterium tuberculosis* complex, affecting more than ten million people worldwide.[1] It causes ill-health for approximately 10 million people each year and is one of the top ten causes of death worldwide. For the past 5 years, it has been the leading cause of death from a single infectious agent, ranking above HIV/AIDS. There are an estimated 79,000 multi-drug resistant TB patients among the notified cases of pulmonary TB each year. India is also the country with the second highest number (after South Africa) of estimated HIV associated TB cases.[2] The increase in HIV infection cases was the most important factor in the growth in TB prevalence rate. Nowadays, the disease is getting more worrying status since resistant cases are rising every day. Despite this, TB resistant cases can be classified in multidrug resistant TB (MDR-TB, when the resistance to first-line agents is detected, including resistance to isoniazid or rifampin) and extensively drug-resistant TB (XDR-TB, when second-line agent resistance is detected).[3]

In addition to providing effective treatment and reducing mortality, a primary aim of tuberculosis (TB) control programs in countries of high TB incidence is to reduce the transmission from infectious TB cases. HIV co-infection greatly increases the chances of reactivation of latent infection of TB and increases the rapid TB progression following primary infection or re-infection with TB.[4]

There is a major global health problem attributable to diseases, such as tuberculosis (TB), which are complicated due to drug resistance. This is coupled with the problem of mycobacterial persistence, thus highlighting the need to develop novel TB drugs that are not only active against drug resistant bacteria, but more importantly, kill persistent bacteria and shorten the length of treatment. With the rising prevalence of microorganisms showing resistance to antibiotics, there is an urgent need to develop new antimicrobial compounds.[5]

Due to an increasing demand for chemical diversity in screening programs, seeking therapeutic drugs from natural products, interest particularly in edible plants has grown throughout the world. Botanicals and herbal preparations for medicinal usage contain various types of bioactive compounds.[6] Traditionally the plant is used as laxative, purgative, fertilizer and fungicide etc. Plant possess beneficial effects such as anti-oxidant[7], antihistamnic, antinociceptive, antiasthmatic, antiulcer, immunemodulatory, antidiabetic[8], hepatoprotective[9], antifertility[10], antiinflammatory[11-12], antimicrobial, central nervous system stimulant, lipolytic[13], wound healing, insecticidal and Larvicidal and many other medicinal properties.[14] *Ricinus communis* Linn. seeds are widely used in African folk medicine for the treatment of malaria, fever, stomach-ache, coughs, sexually

transmitted diseases, tooth ache, breast cancer and constipation.[15] The seed has been found to be effective in the treatment of haemorrhoids, jaundice, ulcer, headache, sores, epilepsy, rheumatism and sciatica.[16] Natural products, or their semi-synthetic derivatives, well defined as providing novel examples of anti-infective drug leads, currently play important roles in the chemotherapy of tuberculosis.[17]

Despite the popular use of *Ricinus communis Linn.* as a medicinal plant, no data have been published on the anti-tubercular activity of plant and chemical constituents of leaves extracts of this species. Earlier in research papers we have reported the anti-tubercular activity against std. strain of *M. tuberculosis* H37RV and the phytochemicals of the ethanol and other different extracts of *Ricinus communis* Linn. Leaves.[18-19] The present study was undertaken to investigate the antitubercular activity of various bioactive compounds obtained from ethanol extract of *Ricinus communis Linn.* and isolation and characterization of some bioactive compounds.

MATERIALS AND METHODS

General and Chemistry

Isolation and identification was monitored on silica gel G coated TLC plates in the different solvent system. Compounds on TLC were spotted either by exposing to iodine vapors or by spraying 40% sulphuric acid. UV-spectrophotometer (Jasco V550), HPTLC system (Camag with Wincat, Anchrome lab, Mumbai) used in study. Standard oleic acid and linoleic acid purchased from Sigma-aldrich, Mumbai, India. Column chromatography was carried out on silica gel 60 (60-120 mesh, 0.063–0.200 mm) obtained from Merck and Infrared (IR) spectra were recorded as thin film (for oils) in NaCl discs with a JASCO FT/IR-410 Spectrometer and values are represented in cm^{-1}. ^{1}HNMR spectra recorded on a Bruker model AV 300MHz in CDCl3 acquired with a Varian INOVA 500 spectrometer; chemical shift (δ) values are reported in parts per million (ppm) using tetramethylsilane (TMS) as internal standard. The abbreviations used to describe the splitting pattern are as follows: s for singlet; d for doublet; dd for double doublet; t for triplet; m for multiplet. GC-MS was recorded on (Hewlett Packard, GCD: 1800 A.) Rifampicin and isoniazide were obtained as gift sample from Lupin, Mumbai. The standard strain of bacteria *M. tuberculosis* H37RV sensitive to all the anti-tuberculosis agents procured from Jalma institute of Leprosy and other mycobacterial diseases, Agra. Loweinstein-Jensen (LJ) media and McCartney bottles purchased from Himedia, Mumbai. BacT/Alert 3D system (Biomerieux, France) was used for evaluation of antitubercular activity. All other chemicals and regents used in the work were of analytical grade and purchased from (E Merck, Mumbai) India.

Antitubercular activity

Antitubercular activity was carried out and results were reported for different extracts against Standard strain *Mycobacterium tuberculosis H37RV* by Proportion method[18-19], Nitrate reductase assay (NRA) and BacT/Alert method and reported that 200 µg/ml ethanolic extract of *Ricinus communis* Linn. is sensitive to *M. tuberculosis* H37RV.[19] Therefore, the ethanolic extract was also screened for anti-tubercular activity by resistance ratio method.

Resistance ratio method

The resistance ratio (RR) method utilizes the ratio of the minimum inhibitory concentration (MIC) for the patient's strain to the MIC of the drug-susceptible reference strain, *M. tuberculosis* H37RV, both tested in the same experiment. Inclusion of the reference strain in each experiment is not only for quality control but also to standardize the results by taking into account the test variations within certain permissible limits. Readings were taken after 4 weeks of incubation for the results. Growth is defined as the presence of 20 or more colonies after four weeks of incubation and MIC is defined as the lowest drug concentration in the presence of which the number of colonies is less than 20.[20-22] For preparation of inoculum from patient strain, sputum was collected and decontaminated by Petroff's method.[20] After decontamination sediment was inoculated on solid LJ media in McCartney bottles. Two to three colonies from pure culture of standard strain of *M. tuberculosis* H37RV were inoculated on solid LJ media. Both cultures are incubated at 37^0C until growth of colonies was observed and then examined for morphological characteristics of *Mycobacterium tuberculosis* and confirmed by acid-fast staining.[23] Extract samples were incorporated in LJ media in such a way that final concentrations obtained were 100 and 150 µg/ml of ethanol extract of *Ricinus communis* Linn. For each one concentration of extract 3 bottles were used for inoculation of patient's strain and other three were used for standard strain of *Mycobacterium tuberculosis* H37RV.With the help of wire loop of 2.5 mm internal diameter Streak a loopful of the bacterial suspension on the control slope and test extract sample. This inoculum consist of about 10^4 - 10^5 bacterial aggregates per slope. All the bottles were kept for incubation at 37^0C for a period of 4 weeks. The colonies were counted for each test extract samples in all bottles inoculated with patient strain and standard strain separately. The result was expressed as a resistance ratio by comparison with a control culture as follows.

$$\text{Resistance ratio} = \frac{\text{Lowest conc.of drug that inhibits the patient strain of Mycobacterium tubercle}}{\text{Lowest Concentration of standard drug sensitive strain H 37 RV}}$$

Strains were considered to be resistant to test extract sample if the resistance ratio was 8 or more. If it was 4 is suggestive of resistance but not conclusive and if it was 2 then it was sensitive.[20-22, 24-25]

Isolation and Characterization

Column chromatographic separation

On the basis of phytochemical analysis and TLC examination, ethanol extract subjected to column chromatography over Merck silica gel 60 (130g, 60-120 mesh). Elution of the column was performed to collect total lipids sequentially with Chloroform, acetone and finally methanol. A total of 12 (1 to 12) fractions of neutral lipids were eluted by chloroform. On the same column eluted the total 20 (13 to 32) fractions of glycolipids by acetone and finally 16 (33to 48) fractions of phospholipids eluted by methanol.

TLC of fractions

A total of 48 fractions were collected from column chromatography and TLC of each fraction was carried out and detected with 40% sulphuric acid to find Rf. TLC of neutral lipids by solvent system N-hexane/diethyl ether/acetic acid (60:40:0.1 v/v/v) while the glycolipid and phospholipids subclasses by 25% chloroform/methanol/ammonia solution (65:25:4 v/v/v). Fractions showed same identical λmax and Rf were combined and labelled as CF-1 and CF-2 from chloroform. A total of 6 compounds from acetone labelled as AF-1 to AF-6 and 5 from methanol labelled as MF-1 to MF-5.

Anti-tubercular activity of isolated compounds by NRA

Antitubercular activity of five isolated compounds collected from column chromatography was performed by NRA.[26,27] In the present study, NRA which is based on the ability of *M. tuberculosis* to reduce nitrate to nitrite, the reduction detected by using specific reagents which produces a colour change. The critical concentrations used were 0.2µg/ml for Isoniazid, 40µg/ml for rifampicin standard and 50 and 100µg/ml concentrations of isolated compounds. The LJ media prepared according to procedure described in proportion method.[22] The concentration of KNO_3 (1 mg/ml) was used in the media[26,27] but however growth was not observed of *M. tuberculosis* H37RV (in the form of pink colour) in control bottles. Hence method was modified as KNO_3 (30 mg/ml) instead of 1mg/ml was added to the media and growth was observed of *M. tuberculosis* H37RV (in the form of pink colour) in control bottles. In all control bottles reddish/violet coloration was observed on the surface of the slants indicative of a positive NRA. No reddish/violet coloration was observed on the surface of the slants for reference std. rifampicin, INH and some isolated compounds, absence of colouration interpreted as negative NRA. The results were obtained after seven days of inoculation in all the control, std. and test sample containing bottles.

Characterization

TLC and HPTLC analysis of isolated compounds

TLC of ethanolic extract and std. Linoleic acid was carried out in Mobile phase Acetonitrile:water (9.5:2.5) and spots were detected with Iodine vapours. Isolated and dried compound CF-1 subjected to TLC in mobile phases Benzene: Ethyl acetate (9.1:0.9) and compound CF-2 in Hexane: diethyl ether: acetic acid (6:4:0.1) and spots were detected by iodine vapours. HPTLC of all isolated five compounds were carried out. HPTLC of std. oleic acid and linoleic acid with ethanol extract were performed. The ethanolic extract and isolated compound CF-1 subjected for HPTLC analysis in n-hexane/diethyl ether/ acetic acid (60:40:0.1 v/v/v) and detected by iodine vapours. Photo documentation of TLC plate image scanned at 254 nm was done after derivatisation. Oleic and Linoleic acid was used as working standard for quantification of content in extracts. HPTLC of std. Linoleic acid with ethanol extract was performed.

Spectral data

The isolated Compound CF-1 and CF-2 was single component identified from TLC, HPTLC analysis and others were mixture of components. The structure of CF-1 and CF-2 were elucidated using spectroscopy methods including UV, IR, ^{1}HNMR[28], GC-MS and structures were confirmed by comparison against literature spectroscopic data.[29]

RESULTS AND DISCUSSION

Currently, the increased number of multidrug-resistant (MDR-TB), extensively-drug resistant (XDR-TB) and in some recent reports, totally drug-resistant TB (TDR-TB) cases raises concerns about this disease. MDR-TB and XDR-TB have lower cure rates and higher mortality levels due to treatment problems.[28] The result was expressed as a resistance ratio by comparison with a control culture. Fourteen numbers of colonies of *M. tuberculosis* H37RV were observed for ethanolic extract of *Ricinus communis* Linn. at 100 µg/ml while ten number of colonies were observed for patient strain at 150 µg/ml. Therefore MIC for standard strain and patient strain is 100 µg/ml and 150 µg/ml respectively. The resistance ratio is found to be 1.5 since the value of resistance ratio (RR) is less than 2 which indicates *M. tuberculosis* is sensitive to the ethanolic extract. Therefore it is concluded that ethanolic extract is sensitive to *M. tuberculosis* by resistance ratio method.

A total of five compounds isolated and collected from column chromatography of ethanolic extract of *Ricinus communis* Linn. were screened for anti-tubercular activity by NRA method.

The results of table 1 revealed that CF-1, CF-2, C-6 and M-5 inhibit the growth of *M. tuberculosis* which is interpreted as –ve NRA while A-5 did not inhibit the growth of *M. tuberculosis* which is interpreted as +ve NRA. Oleic acid (CF-1) was having highly anti-tubercular activity at MIC of 50 µg/ml while linoleic acid (CF-2) showed at MIC of 100 µg/ml. M-5 posses anti-tubercular activity at 100 µg/ml while C-6 at 50 and 100 µg/ml but its structure elucidation could not be confirmed by IR, NMR

and GC-MS as they are mixture of more than one constituents showed by TLC.

Table 1: Effects of isolated compounds from ethanolic extract of plant on *M.tuberculosis* by NRA method.

Sr. no.	Name of the isolated compounds	Concentration (µg/ml)	Growth of *M. tuberculosis* indicated by reddish/violet colouration		
			I	II	III
1	Control without drug	--	+	+	+
2	Rifampicin	40	--	--	--
3	Isoniazide	0.2	--	--	--
4	Compound CF-1	50	--	--	--
		100	--	--	--
5	Compound CF-2	50	+	+	+
		100	--	--	--
6	Compound C-6	50	--	--	--
		100	--	--	--
7	Compound A-5	50	+	+	+
		100	+	+	+
8	Compound M-5	50	+	+	+
		100	--	--	--

Note: -- indicates no change in colour means no growth of *M. tuberculosis*

\+ indicates development of reddish violet colour means growth of *M. tuberculosis*

Ethanolic extract showed single dark yellowish green smearing spot on TLC plate in acetonitrile and water (9.5: 2.5) at Rf value 0.85 and identified and confirmed as linoleic acid since standard linoleic acid was having same Rf value 0.85.

After confirmation of its presence by TLC, extract was subjected for isolation of fatty acids by column chromatography. Isolated CF-1 fraction showed single yellowish spot on TLC plate in benzene and ethyl acetate (9.1: 0.9) at Rf value 0.53 and identified and confirmed as oleic acid since standard oleic acid was having same Rf value 0.53. Isolated compound CF-2 matches to std. Linoleic acid (Rf value 0.85). Among all the fractions CF-1 and CF-2 fraction obtained showed single spot and identified as oleic acid and linoleic acid respectively.

Both CF-1 and CF-2 subjected to HPTLC study. HPTLC study of isolated CF-1 (Oleic acid) confirmed its presence in ethanolic extract. Figure 1 showed similar Rf value 0.40 for isolated compound CF-1 as observed in the ethanolic extract indicating presence of CF-1 in the ethanolic extract. The content of CF-2 (linoleic acid) in ethanolic extract was calculated by using HPTLC and was found to be 2.501% and isolated linoleic acid content was found to be 53.8 %.

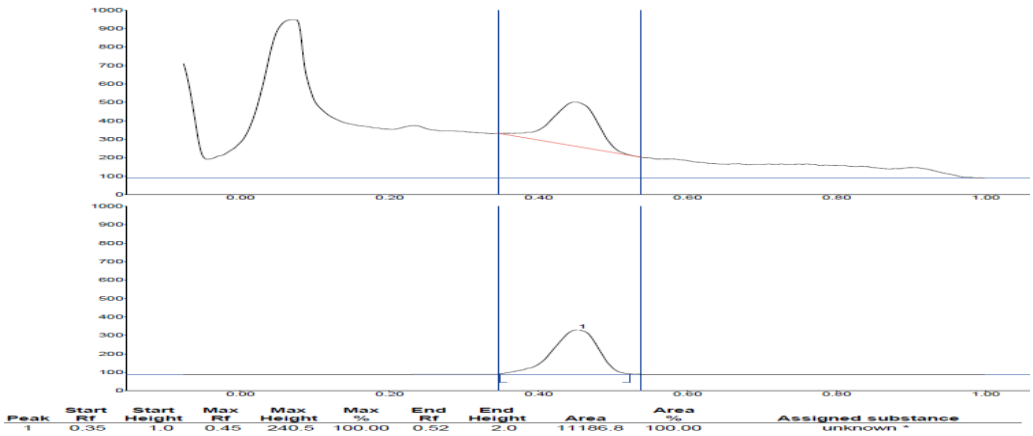


Figure 1: HPTLC study of ethanolic extract of *Ricinus communis* Linn. and isolated CF-1.

The UV spectrum of CF-1 and std. oleic acid showed characteristic peak at λ= 200 nm. and isolated compound CF-2 and std. Linoleic acid showed characteristic peak at λ= 290 nm. UV spectrum of isolated CF-1 and CF-2

compound exactly matches with standard oleic acid and linoleic acid respectively.

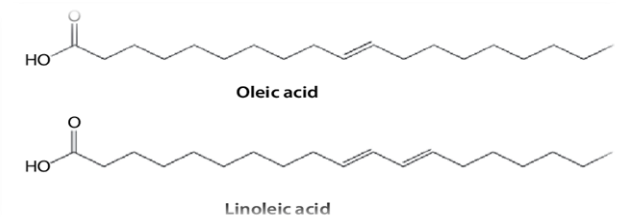


Figure 2: The chemical Structure of Oleic acid and Linoleic acid

Spectral data: Oleic acid: 9(z)-Octadecanoic acid. Molecular formula $C_{18}H_{34}O_2$, Molecular wt. 282.4614g/mol. Faint Yellowish viscous liquid, Yield 74% Rf 0.65 (TLC and HPTLC , petroleum ether :ethyl acetate 4:1). IRvmax cm^{-1}: 3439.88(alcoholic-OH), 1189.86(free-OH), 1739.05(C=O), 2857.37 (C-H), 2923.12 (-C-H) 1266.82 (C-O) ^{1}H NMR (300 MHz, CDCl3): δ=5.134(s, 1H,CH), δH 5.376 (s,2H,CH=), δH 1.347 (m,3H CH3), δH 2.056 (m,14H, 7xCH2), δH 2.301 (d, 2H, CH_2), δH 1.269(m,14H, 7xCH2), δH 11.20(s,1H,COOH).

Linoleic acid: 9, 12-octadecadienoic acid. Molecular formula $C_{18}H_{32}O_2$, Molecular wt. 280.4455 g/mol Dark yellow viscous oil, Yield: 70%, IRvmax cm^{-1}: 3442.31(alcoholic-OH), 1384.64(C-O stretch, carboxylic), 1635.34 (C=O), 2922 (-C-H), ^{1}H NMR (300 MHz, CDCl3) δ= 1.284 (m, 7H, 3xCH2), δH 5.34 (s,1H,CH), δH 5.362 (s,1H,CH=CH), δH 5.374 (s,1H,=CH), δH 2.807(t, 2H, CH_2), 1.255 (m,4H 2xCH2), δH 2.006 (d, 2H, CH_2), δH 0.854(t,3H, CH_3), δH 11.20 (s,1H,COOH).

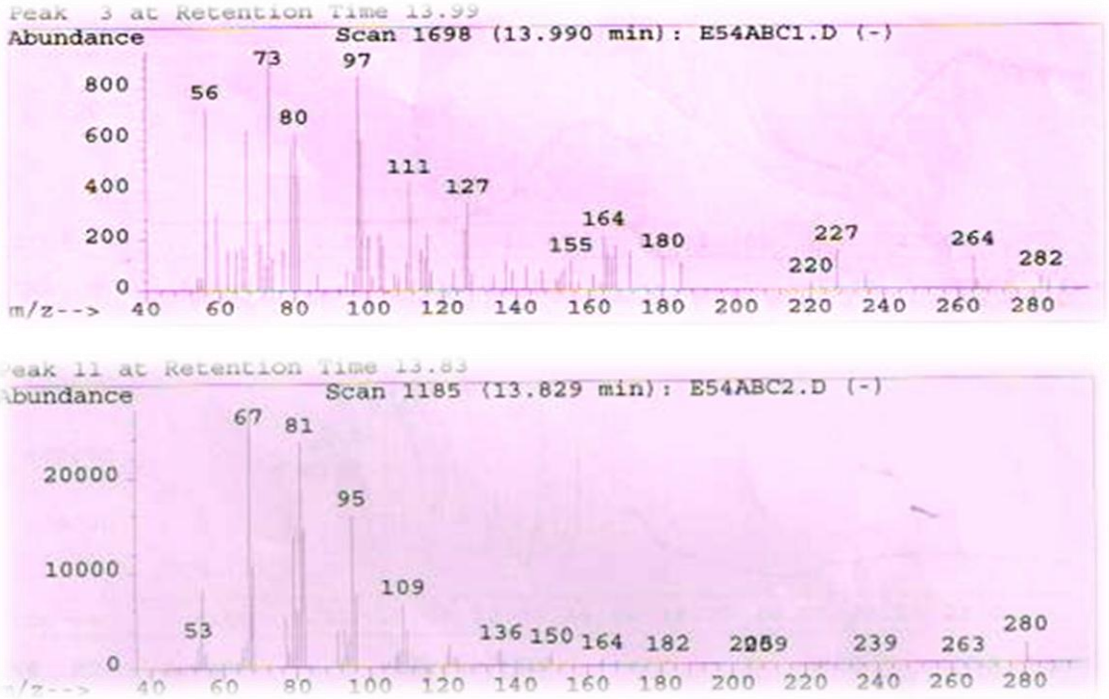
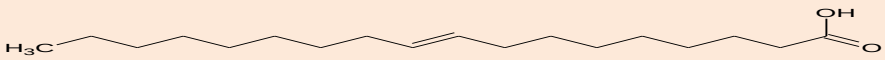
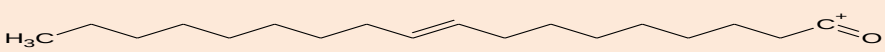


Figure 3: GC-MS analysis (*rel.int%*):*m/z* of oleic acid and Linoleic acid

Table 2: Fragmentation pattern of isolated (CF-1) Oleic acid by GC-MS

m/e	Justification for fragment
282	
265	
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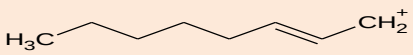
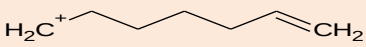
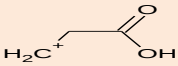
111	
97	
73	
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Table 3: Fragmentation pattern of isolated (CF-2) Linoleic acid by GC-MS

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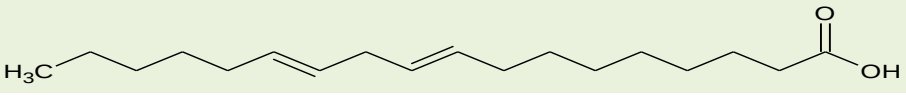
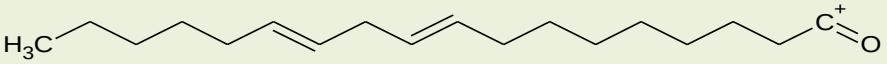
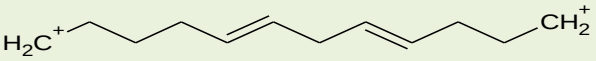
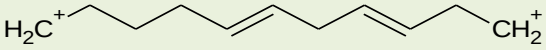
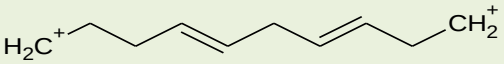
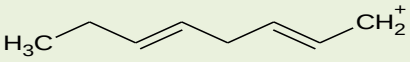
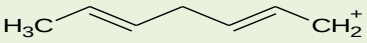
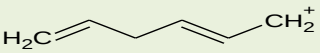
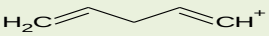
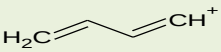
280	
263	
164	
150	
136	
109	
95	
81	
67	
53	

The structure of CF-1 and CF-2 were elucidated using spectroscopy methods including UV, IR, ^{1}HNMR[29], GC-MS and structures were confirmed by comparison against literature spectroscopic data.[30]

CONCLUSION

Emergence MDR-TB is posing an increased threat to TB control programs and HIV fuels the TB epidemic. Results concluded that *Ricinus communis* Linn. has remarkable anti-tubercular activity. Oleic and linoleic acid with significant anti-TB potential and the most active compounds could be useful as a template for treatment of tuberculosis in future. More definite studies in-vivo in the animals susceptible to tuberculosis required in future to define the mechanism of action of these compounds.

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