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Research Article

ALLIUM SATIVUM LINN. CONTAINS LINEAR ALKYLBENZENE SULFONATES THAT ALTER MEMBRANE FLUIDITY FOR THE INHIBITION OF *MYCOBACTERIUM TUBERCULOSIS* H37RA

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ABSTRACT

Objectives: The purpose of the study is to characterize antimycobacterial phytoconstituent from ethyl acetate extract of dried bulbs of *Allium sativum* Linn. (Alliaceae) and elucidating the probable mode of action of the bioactive molecule.

Methods: Serial extraction, *Mycobacterium tuberculosis* assay by agar well diffusion method, minimal inhibitory concentration by microplate alamar blue assay, Fourier transform infrared (FT-IR), nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography (LC)-electrospray ionization (ESI)-mass spectrometry (MS)/MS, cell leakage assay, scanning electron microscopy (SEM), inhibition property of linear alkylbenzene sulfonate (LAS) in the presence of rifampicin on *M. tuberculosis* were performed.

Results: Ethyl acetate extract displayed significant inhibition properties against *M. tuberculosis* H37Ra (MTCC 300). Subsequently, the bioactivity-guided fractionation was employed to purify the phytochemical. Analysis of FT-IR, LC-MS (ESI), ^{1}H, and ^{13}C-NMR spectrum revealed that the bioactive phytochemicals are the variants of LAS, with C_{12}-alkyl being predominant, and the minimum inhibitory concentration was found to be 5.56 µg/ml. Morphological examination by SEM and cell leakage assay indicated that these molecules change the membrane fluidity.

Conclusion: The results thus suggest the possibility of using low concentrations of LAS to effect changes in membrane fluidity, thereby enhancing the efficacy of antibiotic treatment.

Keywords: Bioactivity-guided fractionation, Microplate alamar blue assay, Liquid chromatography-mass spectrometry, Cell leakage assay, Scanning electron microscope.

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INTRODUCTION

Tuberculosis (TB) is one of the most pandemic communicable diseases and accounts for the leading cause of death worldwide. In 2014, about 9.6 million people developed TB, of whom 1.5 million died [1]. Failure to adapt effective strategies for the management of multidrug-resistant TB epidemics will be catastrophic for many countries in the years to come [2]. Drugs that are used to treat TB for the past century (year of discovery given in brackets) are streptomycin (1943), para-aminosalicylic acid (1948), thiacetazone (1951), isoniazid (1952), pyrazinamide (1954), cycloserine (1955), kanamycin (1957), ethionamide (1960), ethambutol (1961), capreomycin (1963), rifampicin (1963), ofloxacin (1982), gatifloxacin (1992), and moxifloxacin (1996). Currently, a few of the prospective drugs are in clinical trials [3]. Long therapy regimen employed to combat TB has been envisaged to be the prime cause for the emergence of drug-resistant TB strains [4,5]. This necessitates the need for exploring alternate medicines from medicinal plants. Plant-based drugs have been used worldwide for the treatment of various diseases [6-8]. In addition, there is a plethora of data available on the antimycobacterial activity of various plant extracts [9-11]. Of the many plants investigated, extracts of *Allium sativum* Linn. (Alliaceae) were shown to possess antimycobacterial activity [12-15]. However, many of the phytochemicals, from this common plant, that are responsible for antimycobacterial activity remains to be unraveled. With this in view, the current study was aimed at: (1) Isolating and characterizing the compound(s), with antimycobacterial activity, present in the bulbs of *A. sativum* and (2) elucidating the probable mode of action of the bioactive molecule.

MATERIALS AND METHODS

Plant material

A. sativum (Garlic) bulbs were purchased from the local market (Vellore, Tamil Nadu, India) in September 2011 and authenticated by Dr. K. Madhava Chetty. A specimen (voucher no. 2011) was deposited in the herbarium of Sri Venkateswara University, Tirupati, India. *A. sativum* bulb was pealed, washed with double-distilled water, chopped into small pieces, and air-dried in shade at room temperature for 2 weeks, and then ground and stored in sterile bags at 4°C until use [16].

Serial extraction

The garlic bulb paste was subjected to serial extraction using a Soxhlet apparatus (HiMedia). Extraction (9 hr) was performed sequentially with 250 ml of solvents in the following order: Petroleum ether, chloroform, ethyl acetate, and methanol. The extracts were evaporated to dryness using a rotary evaporator and stored in sterile bottle (covered with aluminum foil) [17].

Column chromatography

Ethyl acetate extract (0.5 g) of *A. sativum* was subjected to column chromatography (54.0 cm×1.5 cm; 15 g silica gel 230-400 mesh) using petroleum ether-ethyl acetate step gradient (10:0 to 0:10). Fractions (P_1-P_7) were pooled and evaporated to dryness using a rotary evaporator (40°C). The dried samples were transferred into tightly capped sterile glass bottles and stored at 4°C until further use [18].

Ultraviolet-visible (UV-vis) absorption spectra
UV-Vis spectra of the fraction were recorded in methanol in the wavelength range of 200-800 nm using Hitachi double-beam (U-2800) spectrophotometer.

High-performance liquid chromatography (HPLC)
The bioactive fraction was analyzed by HPLC system which consisted of LC-8A prominence liquid chromatography pump and Rheodyne type injector fitted with 20 µl capacity fixed loop from Shimadzu Corporation, Japan. Phenomenex Luna (250 mm×4.6 mm) reverse-phase column was used. Methanol:water (60:40) with the flow rate of 1 ml/minute was used [18]. In case of semipreparative HPLC, the loop capacity was 5 ml and a flow rate of 8.6 ml/minute was used. The signals were monitored and processed using LC solution software. The subfractions were collected manually, evaporated to dryness, and stored in a sterile glass bottle with a tight cap at 4°C until further use.

Antimycobacterial assay
Test organism: *Mycobacterium tuberculosis* H37Ra (MTCC 300) was purchased from MTECH, Chandigarh (India). The strain was subcultured periodically as per the specifications.

Solid culture medium
M. tuberculosis H37Ra (MTCC 300) was cultured by suspending 6.2 g of Loewenstein-Jensen medium base in 100 ml of water containing 2 ml glycerol and autoclaved at 15 lbs for 15 minutes. The egg emulsion (50 ml) was mixed with the base to obtain a uniform mixture. The medium was aliquoted (10 ml) in sterile screw capped bottles and was made to coagulate by heating at 85-90°C for 45 minutes (NCIM). After solidification, *M. tuberculosis* was streaked and incubated at room temperature for 4 weeks.

Liquid culture medium
The pure culture was isolated from the H37Ra (MTCC 300) was purchased from MTECH, Chandigarh (India) (LJ medium) and inoculated into 5 ml Middlebrook 7H9 broth base supplemented with 10% (v/v) ADC and 0.2% (v/v) glycerol. The inoculated medium was maintained at 37°C for 48 hrs on a shaker until an OD_{600} of 0.6-0.8 was reached (equivalent to 3×10^7 cfu/ml) [19].

***M. tuberculosis* assay**
To obtain the qualitative information on the antimycobacterial activity of the extract, agar well diffusion assay was performed [20,21]. In all these experiments, rifampicin (5 µg) served as the positive control.

Determination of minimum inhibitory concentration (MIC)
MIC was determined using microplate alamar blue assay (MABA). Freshly cultured medium was aliquoted (180 µl), and 20 µl of the sample (0.25-16 µg/ml) was dispensed. The plate was incubated at 37°C for 48 hrs, and subsequently 10 µl of alamar blue was added. The lowest concentration of the bioactive fraction that could prevent the color change from blue to pink was taken for MIC determination [22-24].

Fourier transform infrared (FT-IR), nuclear magnetic resonance (NMR) spectroscopy
FT-IR spectrum was recorded using a Perkin-Elmer instrument in the range of 400-4000/cm in thallium bromide disc, and absorption peaks in terms of wave numbers (4/cm) were noted. FT-NMR spectra were acquired on Bruker Accent multi nuclei probe spectrophotometer at 400 MHz (^{1}H, ^{13}C). Chemical shifts were expressed as δ value (ppm) using tetramethylsilane as internal standard. The spectra (^{1}H, ^{13}C) were recorded using methanol-D_4 [25].

LC-ESI-MS/MS
The bioactive fraction was subjected to reverse phase liquid chromatography (LC) coupled to electrospray ionization (ESI) tandem mass spectrometry (MS/MS). LC was carried out on Acquity UPLC BEH C18 column (2.1 mm×50 mm, 1.7 µm), using water (H_2O; solvent A) and methanol (MeOH; solvent B), at a flow rate of 0.1 ml/minute. Various gradient elution programs were tried, and 65% B - 75% B for 20 minutes was found to be the optimum gradient. UV absorbance of eluents was monitored at wavelengths of 254 and 280 nm by photodiode array (PDA) detector. Mass spectrometry data acquisitions were done in both positive and negative ion modes; The ESI parameters are as follows: Capillary voltage: −3.5 kV; cone voltage: −30 V; extractor: −5 V; source temperature: 100°C; desolvation temperature: 200°C; cone gas (nitrogen: N2); flow: 50 Lt/h; desolvation gas (N2) flow: 600 Lt/h; LM1, HM1, LM2, and HM2 resolution 15; Argon (Ar) was used as the collision gas for collision-induced dissociation (CID) experiments, i.e., for daughter scans.

Cell leakage assay
The freshly prepared liquid culture (200 ml) of *M. tuberculosis* was aliquoted and centrifuged at 7500 g for 6 minutes for the separation of the bacterial cell (pellet). The pellet was resuspended in 25 ml sterile sodium chloride solution (0.9 g/100 ml) and transferred into a sterile conical flask. The aliquots were mixed with 0, 10, 20, 40, and 80 µg/ml of the bioactive component. Distilled H_2O and NaCl were added as a positive and negative control, respectively. 2 ml of aliquots were removed from each of the suspensions at pre-determined time points (0, 7, 13, 20, 40, 60, 80, and 100 minutes). The samples were centrifuged at 7500 g for 6 minutes, and the supernatant was collected. The leakage of the cells was determined by measuring the OD of the supernatant at 260 nm. 0.9 g/100 ml of NaCl was used as blank [26].

Scanning electron microscopy (SEM)
2 ml of above culture was taken in sterile eppendorf, and centrifugation was done at 7500 g for 6 minutes for the separation of the bacterial cell (pellet). The pellet was dispersed by vortexing into 2 ml sterile sodium chloride solution (0.9 g/100 ml). 20 µl of suspension was taken into cover slip, and staining was done as per the protocol given by Priester *et al*., (2007). This was followed by coating with gold particles and finally visualized under SEM equipped with an energy-dispersive spectral analyzer, Zeiss EV|018, Germany [27]. The image was captured using the parameters 10.0 kV and 20.00 KX of accelerating voltage and magnification, respectively, for all images.

Inhibition property of linear alkylbenzene sulfonate (LAS) in the presence of Rifampicin on *M. tuberculosis*
M. tuberculosis was cultured in 50 ml Middlebrook broth base and incubated at 37°C for 48 hr. Various concentrations of rifampicin (0, 0.025, 0.05, 0.075, 0.10, 0.125, and 0.15 µg) and LAS (1, 2, 3, and 4 µg) were added to the culture (50 µl). Bacterial growth was monitored by observing the OD at 600 nm.

RESULTS AND DISCUSSION

Bioactivity-guided fractionation
Ethyl acetate extract of *A. sativum* was recently shown to possess antimycobacterial activity against *M. tuberculosis* H37Ra [21]. To investigate the nature of the bioactive component in the extract, we embarked on purifying the crude extract by employing bioactivity-guided fractionation. Seven distinct fractions (P_1-P_7) were pooled based on the Rf value and homogeneity observed on the thin-layer chromatography (TLC) plates. Agar well diffusion assay was used to obtain information on the antimycobacterial activity of the fractions. However, it should be pointed out that the results of the agar well diffusion assay are highly dependent on the diffusion coefficient of the compounds and therefore cannot be taken as an absolute method to compare the activity of different compounds [28]. Therefore, in the present investigation, the agar well diffusion assay was used to derive preliminary information on the inhibitory potential of the bioactive component in the fractions. Of the different fractions, only P_7 exhibited inhibition property, indicating the presence of a compound with antimycobacterial activity (Fig. S1a). The yield of this component was apparently the highest (22.4%) compared to the other fractions (Table S1). Further analysis indicated that the active fraction, P_7, contained at least two components (as judged from TLC and HPLC) (Fig. S1b and c), and therefore, subsequent fractionation

was performed by semi-preparative HPLC reverse-phase column. Of the four subfractions ($P_7[F_1]$ to $P_7[F_4]$) collected (Fig. S2), $P_7(F_3)$ and $P_7(F_4)$ exhibited antimycobacterial activity (Fig. S3). However, the ^{1}H-NMR spectral data for both these subfractions ($P_7[F_3]$ and $P_7[F_4]$) were identical. Table 1 summarizes the steps involved in the purification of the active component. The overall yield of the active molecule was found to be 32.0 mg, with a specific inhibitory activity of 2.8 U/mg (overall purification fold of 56).

To determine the minimal inhibitory concentration (MIC) of the purified compound, MABA was performed. The MIC of the active compound is 5.56 µg/ml (Fig. 1a and 1b).

UV, FT-IR, NMR, and LC-MS spectroscopic characteristics of the active component

The bioactive subfraction was found to be odorless light-brownish oil. The UV absorption spectrum (Fig. S4a) displayed a λ_{max} (in MeOH) at 220 nm. The FT-IR spectrum (Fig. S4b) showed absorptions for aliphatic C-H stretches (at 2924 and 2852/cm) and aromatic C=C stretches (1613 and 1452/cm). Sulfonate group S=O symmetric and asymmetric stretching were detected at 1163 and 1344/cm, respectively. Furthermore, the S-O stretches at 894/cm confirm the presence of a sulfonate group. Such spectral characteristics indicated that the compound could be aromatic with aliphatic side chains along with the sulfonate group. To get further confirmation of the nature of bioactive compound, ^{13}C NMR spectra were recorded. The presence of signals in the region between 7.65 ppm and 7.12 ppm in the ^{1}H NMR (Fig. S5a and b) and resonance at 120-130 ppm in the ^{13}C NMR confirmed the presence of the aromatic moiety. Moreover, the peak pattern in the ^{1}H NMR spectrum corresponded to the protons on the aromatic ring having para-substitutions. Signals in the region between 2.0 and 0.75 ppm in ^{1}H NMR spectrum and in the range 20-40 ppm in the ^{13}C NMR spectrum confirmed the presence of the long-chain aliphatic group in the active compound. However, there was no indication of hydroxyl (-OH) and carboxyl (-COOH) protons in the ^{1}H NMR data. UV chromatogram (detected at 280 nm; PDA) and total ion chromatogram (negative ESI) from liquid chromatography-mass spectrometry (LC-MS) runs revealed the presence of four major components in the bioactive fraction (Fig. S6a and b). The peak area values, 11.67%, 29.52%, 38.24%, and 20.57%, respectively, of each of the four components, are indicated in the UV chromatogram. The intense signals in the respective negative ion mass spectrum of the four components were interpreted as deprotonated ($[M-H]^-$) molecular ions, thereby the molecular masses of the corresponding four compounds were inferred to be 297.09 Da, 311.10 Da, 325.16 Da, and 339.22 Da (Fig. S6c). The difference in the molecular masses (298, 312, 326, and 340 Da) of 14 Da could possibly indicate the presence of various lengths of C_{10}, C_{11}, C_{12}, and C_{13} alkyl chains. Subsequently, each of these four molecular ions was taken as a precursor (parent) ions and subjected to CID (daughter scan) to elucidate their molecular structure. An intense peak at m/z 183 was found in each of the MS/MS spectrum of all the four precursor ions, suggestive of a structural fragment that can be common to all four compounds (Fig. S7). Furthermore, a difference of 14 Da between the peaks indicates that they are alkyl variants differing by a methylene group. The mass difference between the precursor ion and the common fragment ion *m/z* 183 aids in ascertaining the number of methylene units ($-CH_2$) in the aliphatic chain (Fig. S8). Thus, the spectral characteristics taken together confirm the presence of LAS. By comparing the ^{1}H NMR spectra of the commercial LAS of molecular mass 326 Da and bioactive fraction, the identity of the LAS variants was further validated (Fig. S9a and b).

Table 1: Purification process of the ethyl acetate extract of *Allium sativum* gives the yield, specific inhibition activity by plate assay, and purification folds

Purification process	Yield (mg)	SIA[a]	Purification fold
Solvent extraction	600	0.05	1
Column chromatography	134	0.62	12.4
Semi-preparative HPLC	32	2.8	56

[a]SIA=Zone of clearance/Amount of compound loaded on the well. SIA: Specific inhibition activity, HPLC: High-performance liquid chromatography

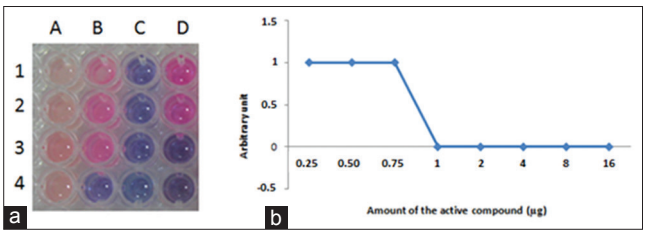

Fig. 1: (a) Graphical representation of the minimum inhibitory concentration (MIC) (1 µg) determination for the bioactive fraction. (b) Microplate alamar blue assay for subfraction $P_7(F_3)$ from ethyl acetate extracts of *A. sativum* bulbs, the MIC was found to be 1 µg. A_1-A_4 are blank, B_1=0.25 µg, B_2=0.5 µg, B_3=0.75 µg, B_4=1 µg, C_1=2 µg, C_2=4 µg, C_3=8 µg, C_4=16 µg, D_1 and D_2 are negative control (dimethyl sulfoxide [20 µl]) D_3 and D_4 are the positive control (rifampicin [5 µg]). This image was taken 24 hr after adding alamar blue. Pink wells represent the absence of inhibition, whereas blue walls indicating the inhibitions of *M. tuberculosis*

Effect of bioactive subfraction $P_7(F_3)$ on growth of *M. tuberculosis*

Morphological examination of the bacterium by SEM indicates changes in the cell membrane in the presence of LAS (Fig. 2a and b). The disruption in the cell membrane was found to increase with an increase in the concentration of LAS used.

To authenticate the changes in the membrane fluidity, time-dependent leakage of cytoplasmic constituents was investigated by measuring the absorbance of the extracellular constituents at 260 nm. It could be found that the absorbance increases as a function of both times as well as LAS concentration, thus indicating that the cell membrane is being disrupted by the phytochemical. Rapid draining of the cytoplasmic constituents can be inferred at higher concentration of LAS, especially when exposed to longer time periods (Fig. 3).

***M. tuberculosis* inhibition property in the presence of LAS with rifampicin**

To ensure the membrane fluidity by LAS, we performed *M. tuberculosis* inhibition assay in the liquid medium with the presence of low concentrations of rifampicin. The mycobacterial growth pattern was clearly distinct (Fig. 4) in the presence and absence of LAS (1, 2, 3 and 4 µg) with lower concentrations of rifampicin (0, 0.025, 0.05, 0.075, 0.1, 0.125, and 0.15 µg). It could be seen that the MIC of rifampicin decreases with increase in the concentration of LAS. These results indicate that LAS may play a role as an adjuvant so as to enhance the

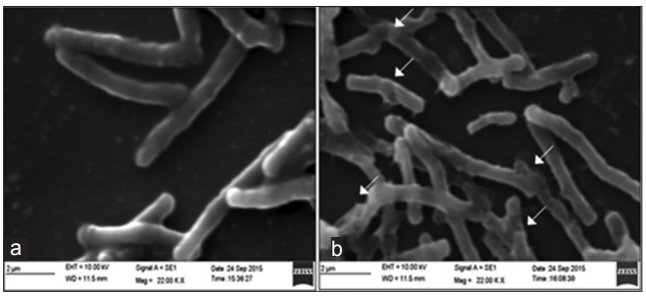

Fig. 2: (a) Scanning electron microscopy illustration shows no changes in the outer cell membrane integrity of *Mycobacterium tuberculosis* with the absence. (b) dramatic cytological modification in *M. tuberculosis* with presence of linear alkylbenzene sulfonate

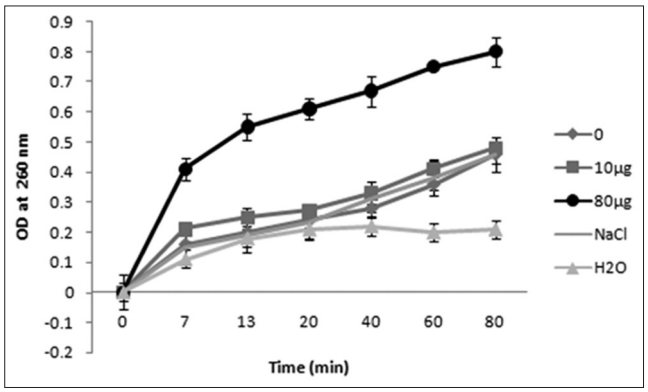

Fig. 3: The plot with respect to the release of cellular materials in the presence and absence of linear alkylbenzene sulfonate (LAS) shows that there was a gradual increase in lower concentration (10 µg/ml of LAS) and in the higher concentration (80 µg/ml of LAS), rapid increases was found

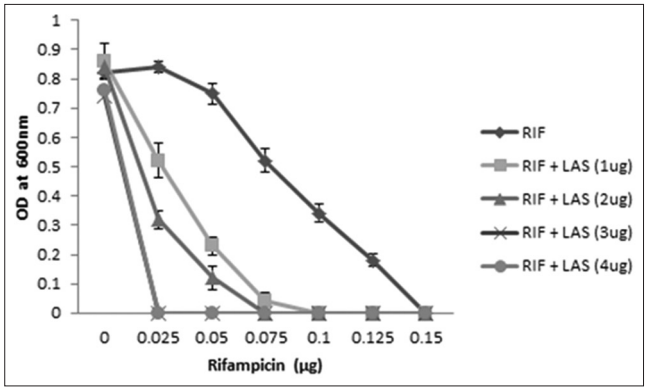

Fig. 4: *M. tuberculosis* inhibition assay in the liquid medium with the presence of LAS (1, 2, 3 and 4 µg) and rifampicin in lower concentrations (0, 0.025, 0.05, 0.075, 0.1, 0.125 and 0.15µg) shows lowering MIC (at 0.025 µg with RIF + LAS) from higher (at range between 0.05-0.075 with only RIF)

effectiveness of antibiotic treatment.

LAS, the anionic surfactant, is rich in the river, sea water, and few natural sources [29]. Certain bacteria are capable of degrading LAS and using them as a sole sulfate source [30]. A study on the *in vitro* germicidal activity of teat dips the linear alkylbenzene sulfonic acid-containing product (1.94%) was shown to be completely effective against suspensions of *Escherichia coli, Staphylococcus aureus*, and *Streptococcus agalactiae*. Hironori, 2015, was exclusively reviewed that the list of antimicrobial drugs and phytochemicals interacting with bacterial cell membranes, resulting in changes of membrane fluidity and permeability [31]. The leaf extract of *Polygonum minus* has changed the cytoplasmic membrane integrity when exposed to *E. coli* and *S. aureus* [32]. Disinfectant quaternary ammonium acts on phospholipids liposomes from *Staphylococcus aureus* and increases the membrane fluidity [33]. Aminoglycoside antibiotics streptomycin and isepamicin are able to interact with negatively charged membranes and increase their permeability [34]. Maurin and Raoult, 2001, were also reviewed that the activity of aminoglycosides (streptomycin, gentamicin, and amikacin) is highly susceptible to *M. tuberculosis* in the axenic medium than that of growing in macrophage which are associated to the fluidity of intracellular membrane [35]. Further Crowle and May, 1990, also demonstrated that alkalinization of *M. tuberculosis*-containing vacuoles using the lysosomotropic agent chloroquine resulted in an expected increased activity of streptomycin [36]. In this study, LAS changes the membrane fluidity, and OD was observed from the leakage of *M. tuberculosis* cell. It facilitates that the antibiotics (Rifampicin) could easily penetrate inside the intracellular plasma membrane. Certainly, there was a decrease in MIC when assayed in combination with Rifampicin and LAS, and thus LAS increases the membrane fluidity, thereby enhancing the effectiveness of antibiotic treatment.

CONCLUSION

As there is a pressing need to unravel alternate medications to combat the evolving drug resistance in *M. tuberculosis*, the present study was focused on unraveling new metabolites from the common Indian herbal plant *A. sativum*. A combination of bioactivity-guided fractionation and spectroscopic analysis of the ethyl acetate extract of *A. sativum* revealed the presence of a secondary metabolite linear alkyl benzene sulfonates. For the first time, LAS has been shown to possess antimycobacterial activity with lower MIC values than any other compounds from *A. sativum*. The primary mode of the action of LAS is to alter the outer cell membrane integrity and break the cell wall. The study thus opens up a possibility for the use of LAS, to combat *M. tuberculosis*.

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SUPPLEMENTARY FILE

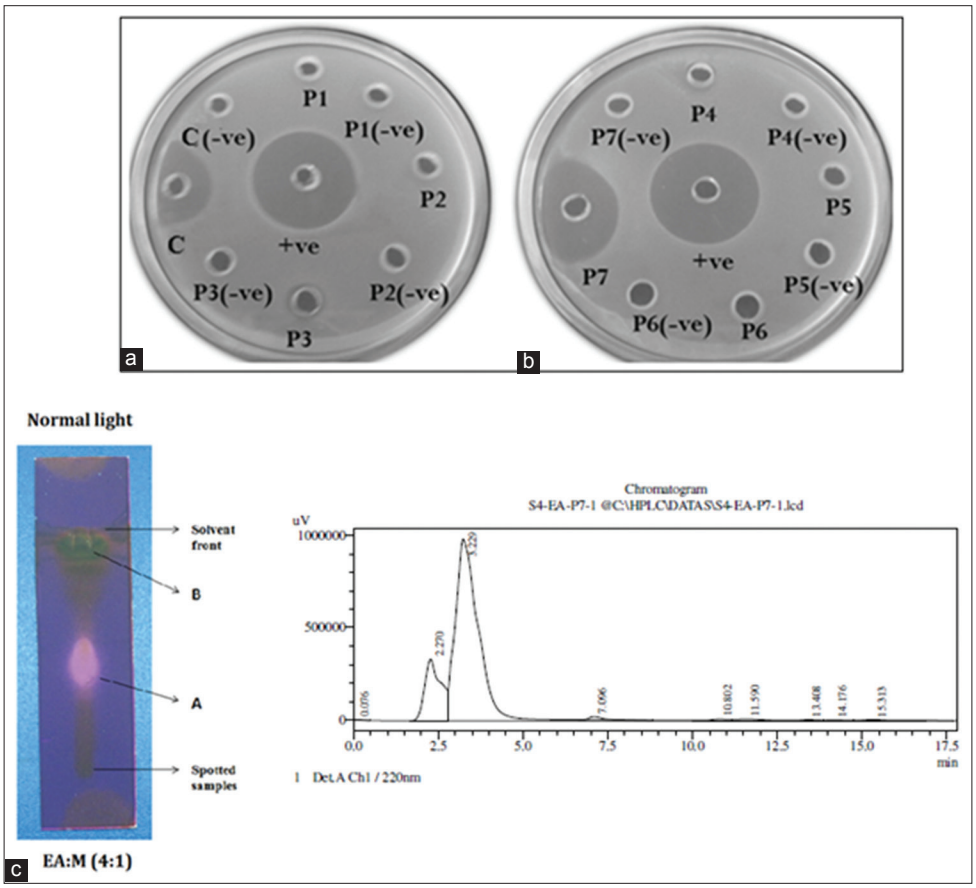
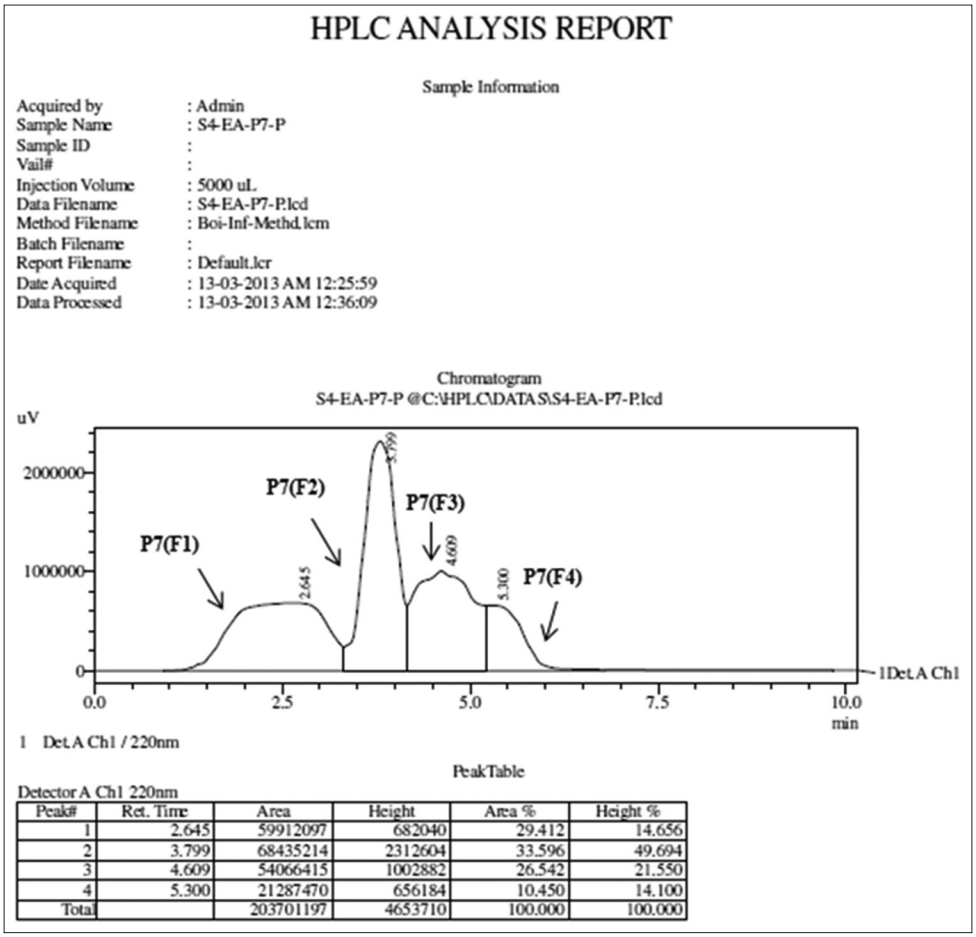


Fig. S1: (a) Antimycobacterial assay for the crude (c) and pooled fractions P1, P2, P3, P4, P5, P6 and P7 of ethyl acetate extract of A. sativum. 50 µl (1 mg/mL) samples were loaded in each well. A clear zone of inhibition is observed only in P7 fraction. (b) thin liquid chromatography shows two spots (a and b) from the ethyl acetate extract of *Allium sativum* (c) the high-performance liquid chromatography spectrum for partially purified fraction (P7)



HPLC ANALYSIS REPORT

Sample Information

Acquired by	: Admin
Sample Name	: S4-EA-P7-P
Sample ID	:
Vail#	:
Injection Volume	: 5000 uL
Data Filename	: S4-EA-P7-P.lcd
Method Filename	: Boi-Inf-Methd.lcm
Batch Filename	:
Report Filename	: Default.lcr
Date Acquired	: 13-03-2013 AM 12:25:59
Data Processed	: 13-03-2013 AM 12:36:09

PeakTable

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.645	59912097	682040	29.412	14.656
2	3.799	68435214	2312604	33.596	49.694
3	4.609	54066415	1002882	26.542	21.550
4	5.300	21287470	656184	10.450	14.100
Total		203701197	4653710	100.000	100.000

Fig. S2: The semi-preparative high-performance liquid chromatography of P7 fraction shows 4 peaks were collected seperately and labeled P7(F1), P7(F2), P7(F3) and P7(F4)

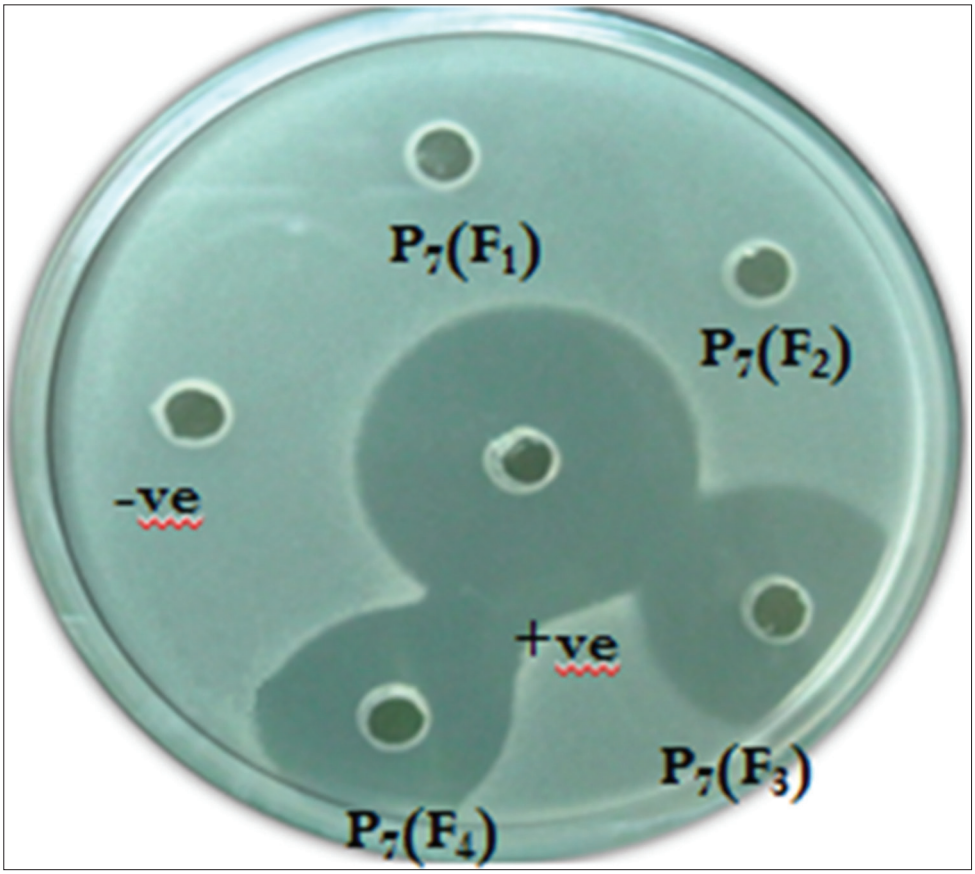


Fig. S3: Among the four fractions zone of clearance was observed in subfractions P7(F3) and P7(F4). Volume of samples loaded = 25 µl (0.5 mg/mL), +ve control = rifampicin (5µl), -ve control = MeOH

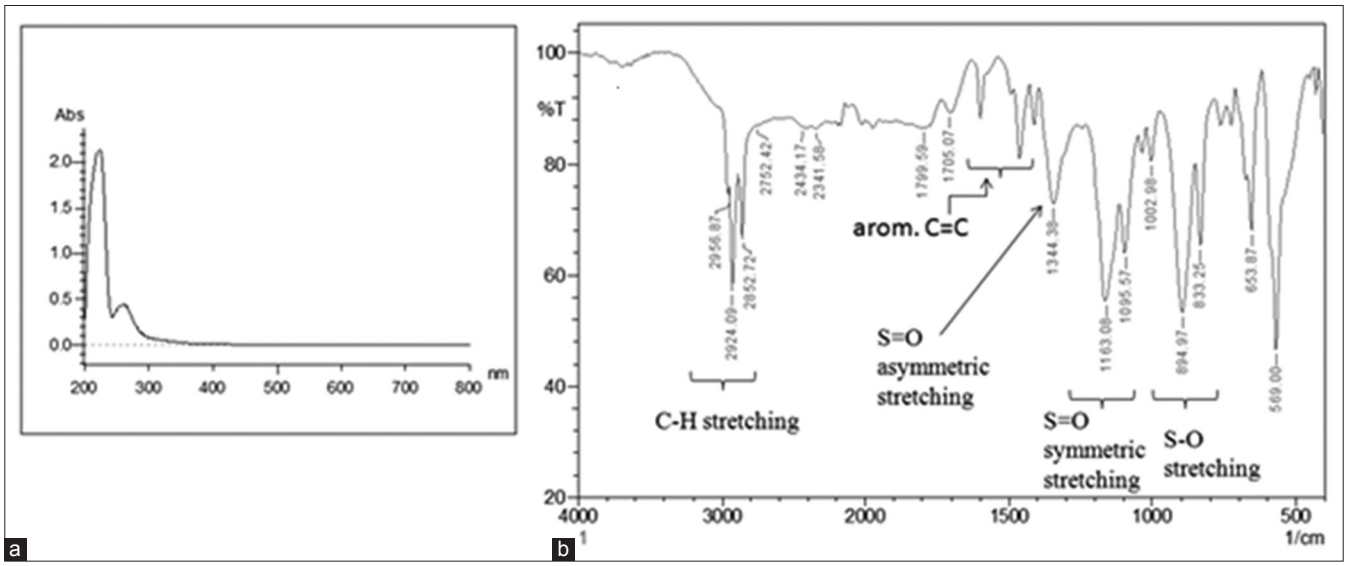


Fig. S4: (a) UV and (b) IR spectrum of the bioactive fraction P7(F3).

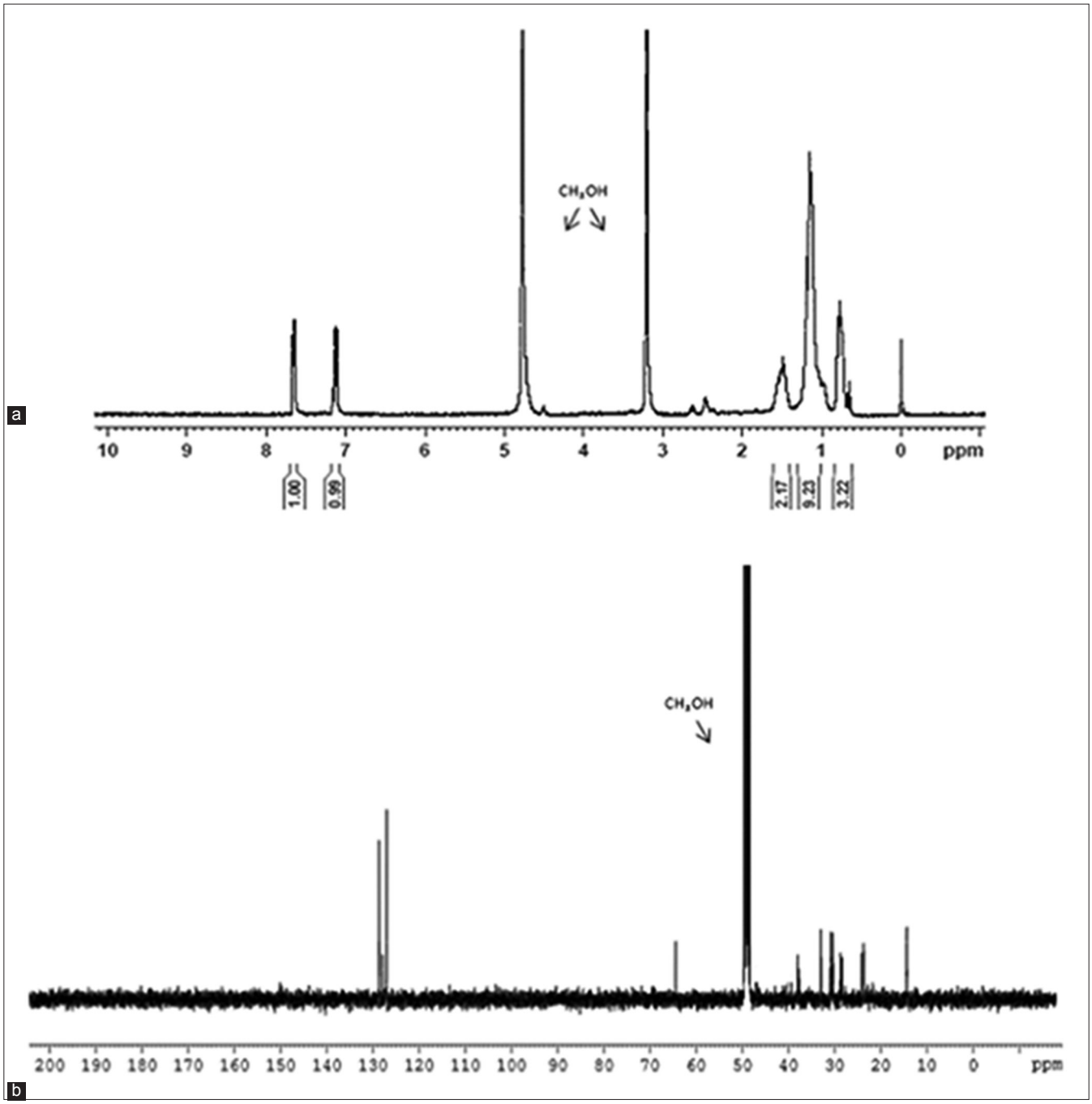


Fig. S5: (a) 1H NMR (b) 13C NMR spectra of the bioactive subfraction P7(F3)

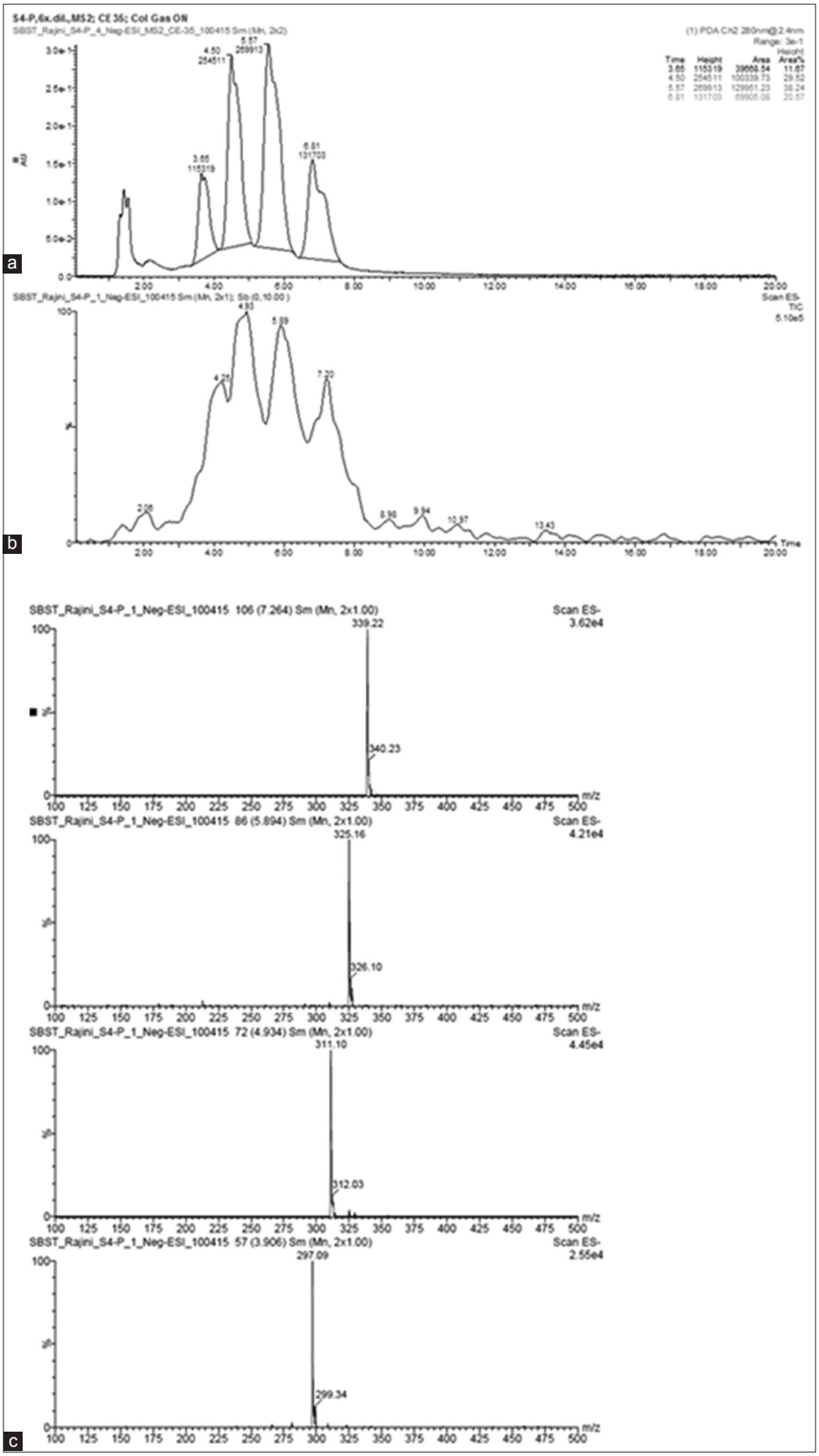


Fig. S6: (a) Representative UV (b) and the total ion (LC-ESI) chromatograms (c) Mass spectrum corresponding to each of the retention time (Rt) of 4.2, 4.9, 5.8 and 7.2 min. showing deprotonated ([M-H]-) molecular ion peak at m/z 297, 311, 325 and 339 respectively, for the bioactive fraction P7(F3)

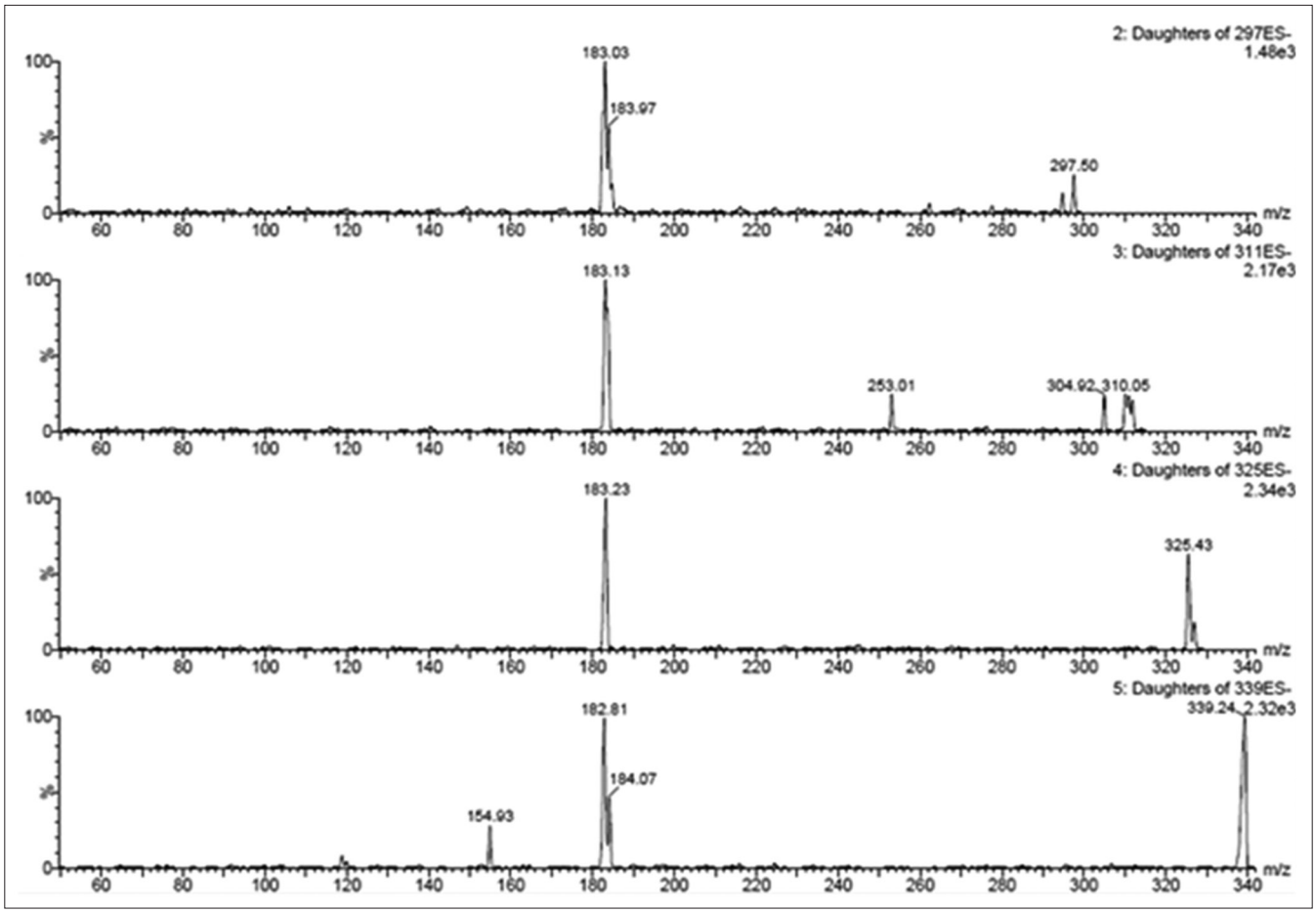


Fig. S7: CID MS/MS spectrum of each of the [M-H]- precursor ion, m/z 297, 311, 325 and 339. The fragment ion peak at m/z 183 that is common across all the four spectra can be noted

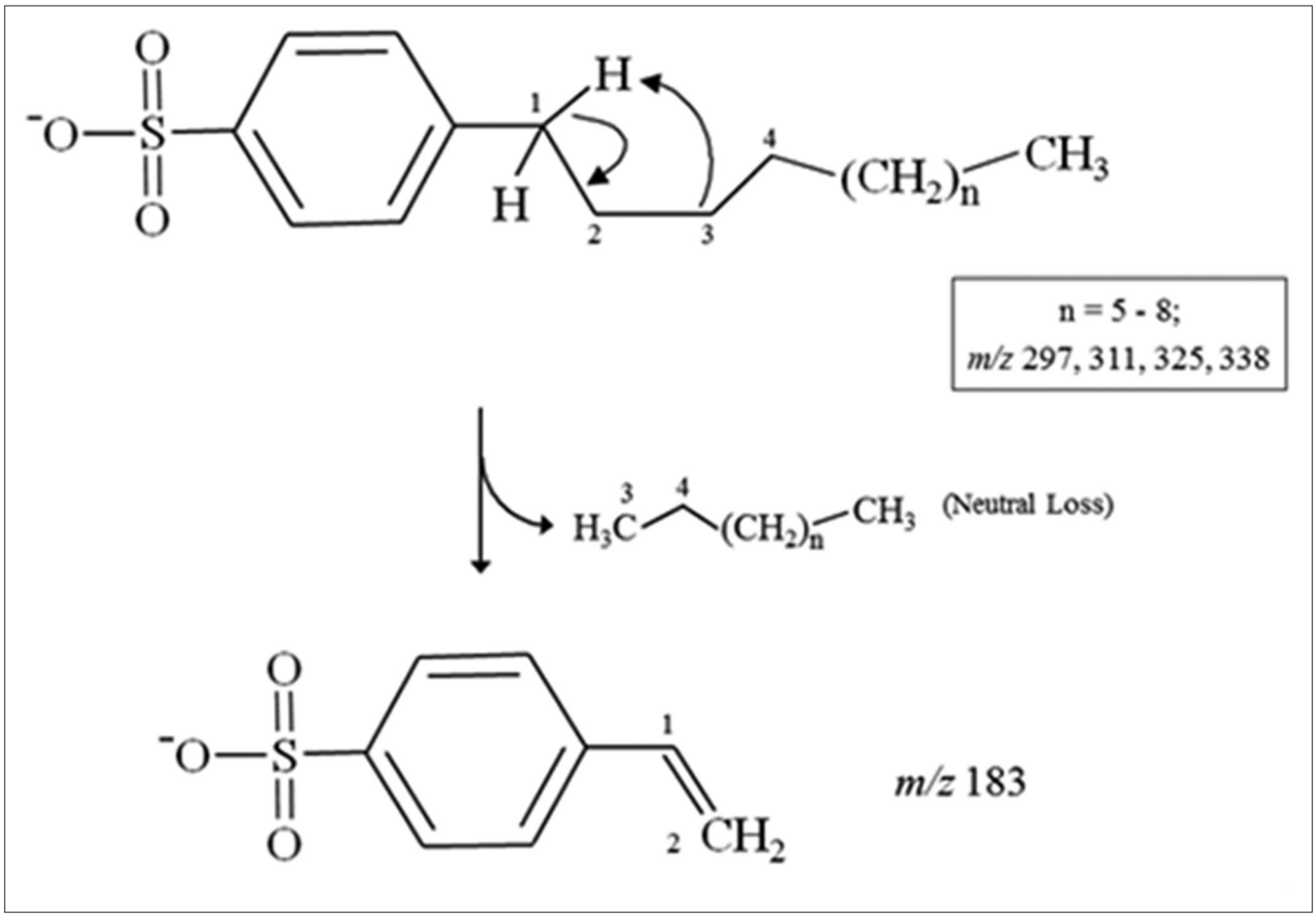


Fig. S8: Plausible mechanism of fragmentation of [M-H]- precursor ions of LAS variants due to CID, rationalizing for the observation of fragment ion peak at m/z 183

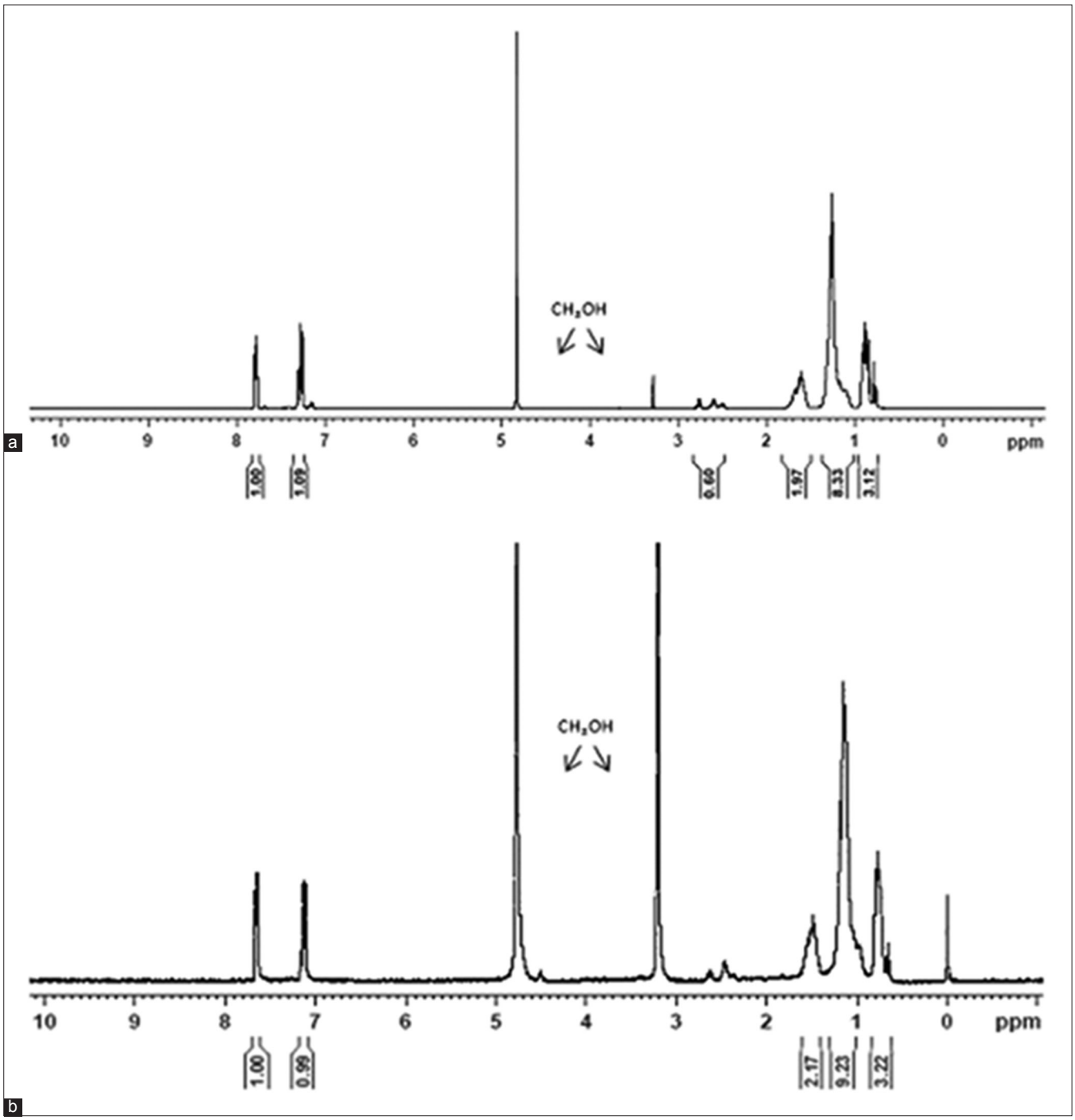


Fig. S9: (a) 1H-NMR comparison between purified (bioactive subfraction P7(F3), (b) commercially available LAS with molecular mass m/z 326

Table S1: Chromatographic fractions of ethyl acetate extract of *A. sativum* of the seven fractions, only P7 exhibited antimycobacterial activity. The retention factor values mentioned is for the TLC plates developed with ethyl acetate: methanol (4:1)

Pooled fractions	Yield (mg)	Yield (%)	Rf value	Antimycobacterial activity
p1	6.2	1.24	0.99	–
p2	64.6	12.92	0.99	–
p3	24.3	4.86	0.99	–
p4	32.4	6.48	0.99	–
p5	26.6	5.32	0.82, 0.99	–
p6	28.8	5.76	0.76, 0.99	–
p7	112.0	22.40	0.01, 0.21	+

+ Presence of activity,
- Absence of activity