Antimycobacterial agents from the essential oil of *Vetiveria zizanioides* (L.) Nash

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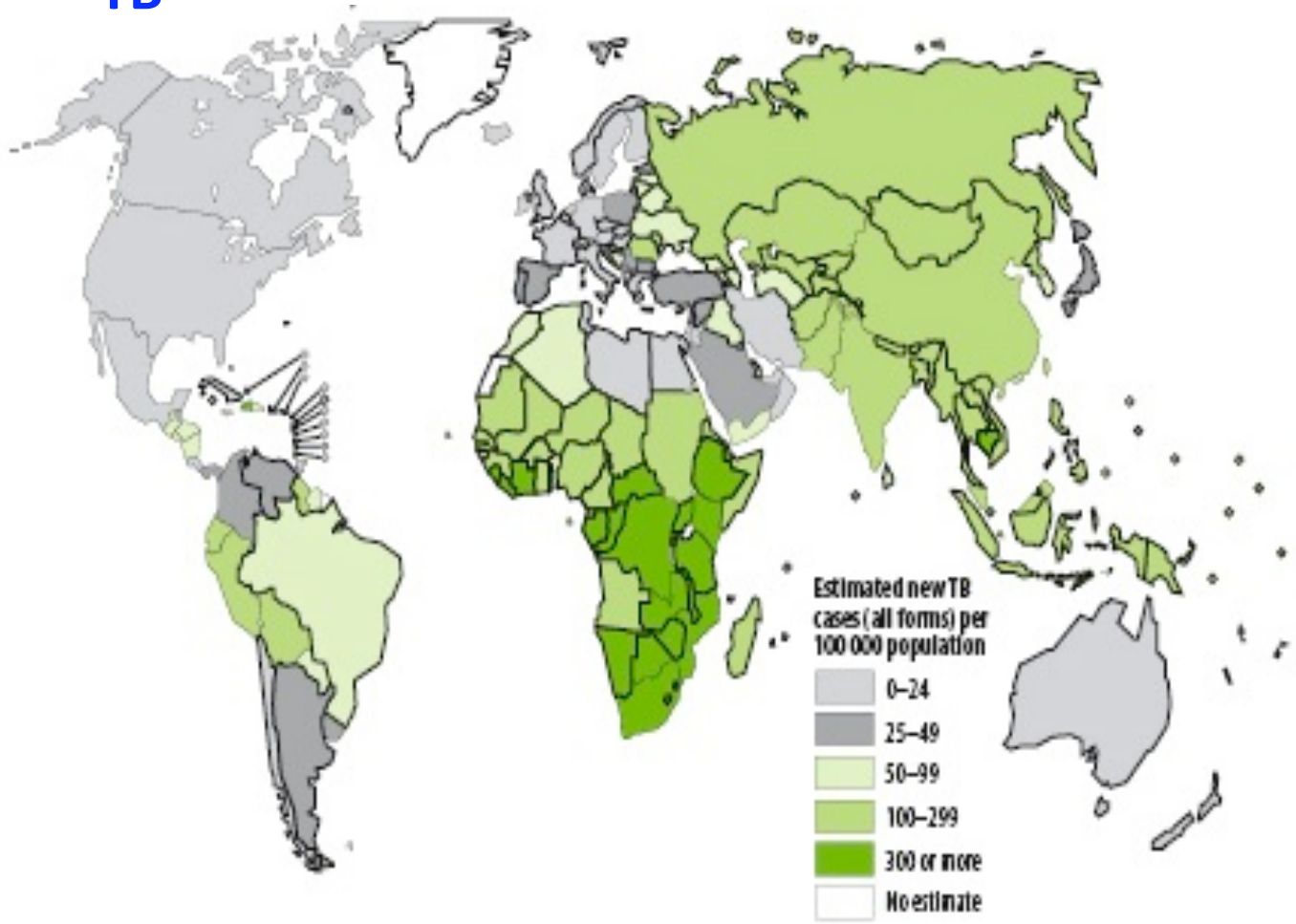
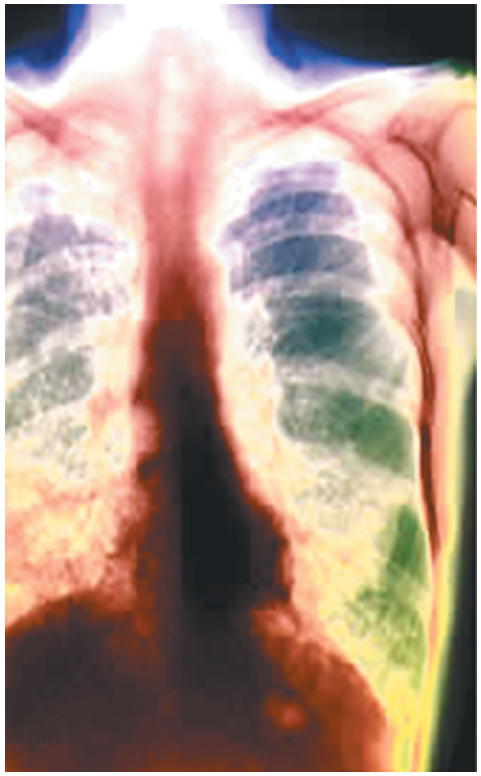
Tuberculosis (TB) is a fatal infectious disease. It is the leading cause of mortality worldwide, infecting about 9 million people and kills approximately 2 million people annually.

These alarming statistics indicates the devastating nature of TB

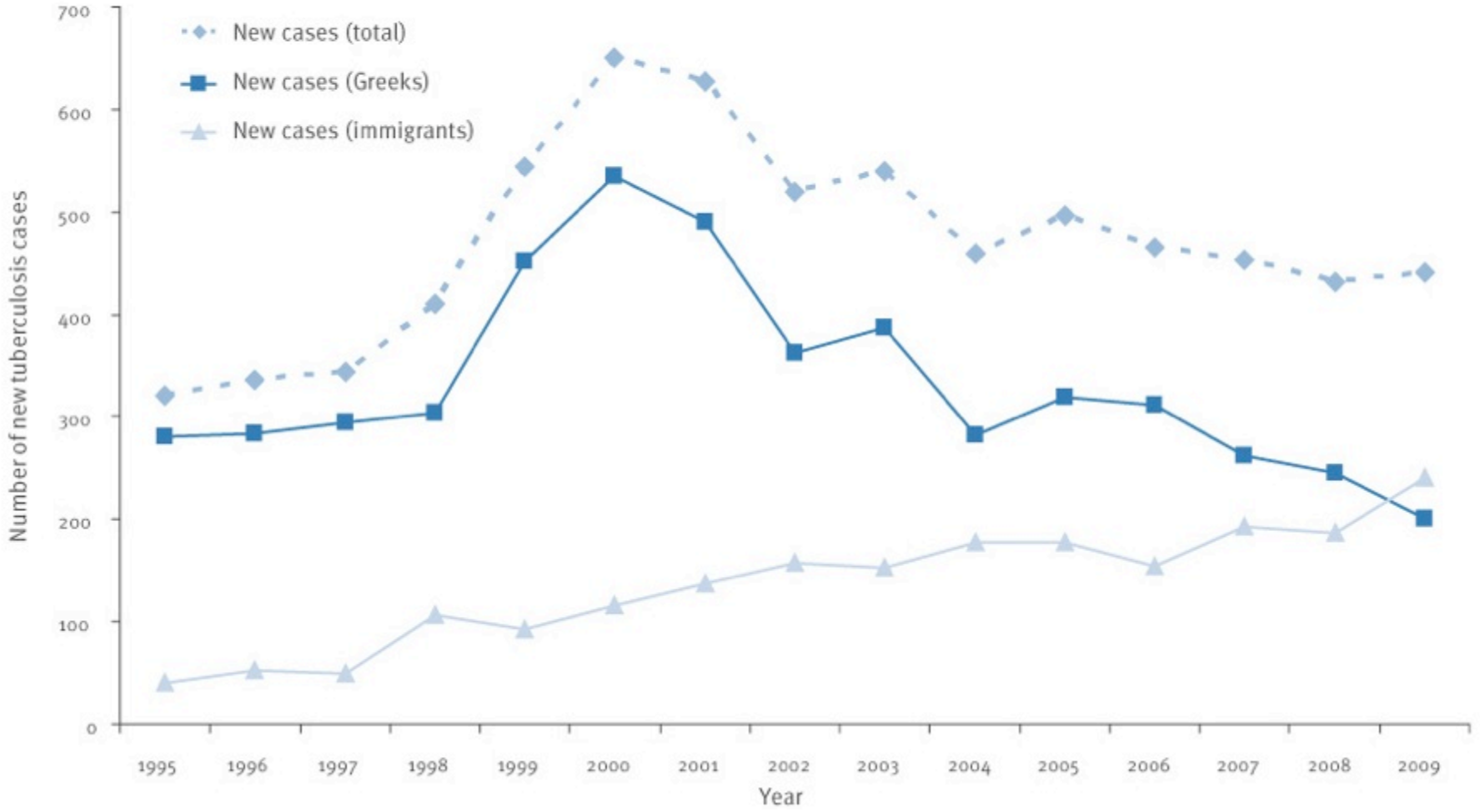




Since 1980s, the number of TB cases throughout the world has been increasing rapidly due to the emergence of multi-drug resistant *Mycobacterium tuberculosis* (MDRTB).

FIGURE 1

Bacteriologically confirmed new tuberculosis cases per year, Greek National Reference Laboratory for Mycobacteria, 1995–2009



- The situation has been recently complicated by the association of TB with HIV in sub-Saharan Africa and many developing countries and also due to the HIV epidemic in many parts of the World.

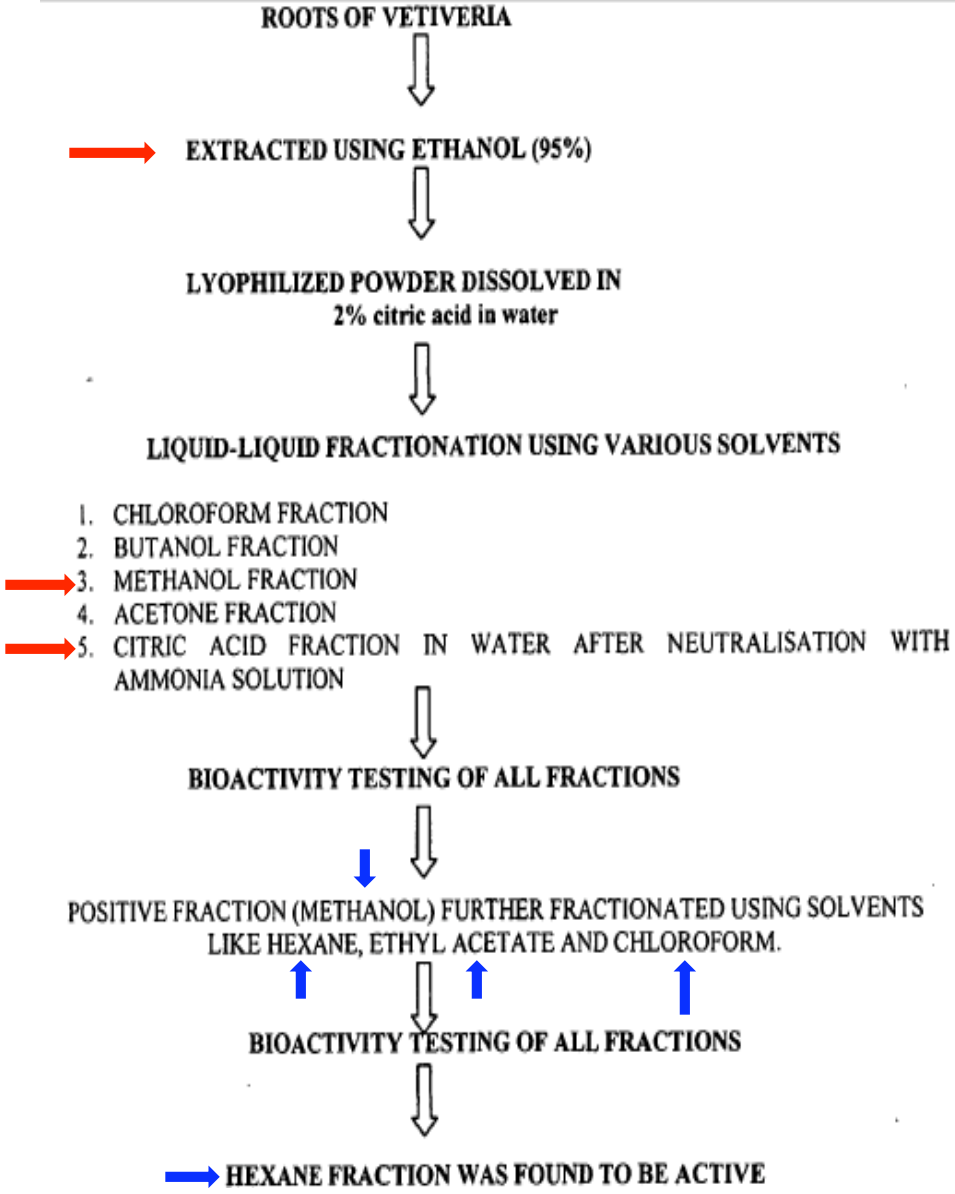
- These forms of the disease are more often fatal and are difficult and expensive to treat.

- Since the past 30 years no anti-TB drug has been introduced, thus, there is an urgent need to search for and develop new, effective, and affordable anti-TB drugs.

- In this scenario, the plant kingdom with enormous chemical diversity may be looked as an important source of new anti-TB agents.

- Of 17,500 higher plant species occurring in India, only about 365 species have been evaluated so far for antimycobacterial activity.

Sometime back our CIMAP scientists invented (US Patent 6,676,974) that methanolic fraction of *Vetiveria zizanioides* roots inhibiting the growth of drug resistant bacterial infections in humans and animals



Bioactivity Response of different solvent fractions of the root extracts of *Vetiveria zizanioides* on different Bacterial Strains added @ 0.8 mg/disc.

Strains	Zone of growth inhibition (mm)		
	Methanol	Spirit	Citric Acid
Salmonella typhimurium	2	2	–
Mycobacterium smegmatis MC^2 155	4	2	1
Pseudomonas aeruginosa	7	6	9
Bacillus subtilis MTCC-121	1		
E.coli CA8000	–	–	–
E.coli DH5	2	–	–
E.coli NK5819	2	–	–
E.coli ET8000	2	–	–

Table-2: Bioactivity response (zone of growth inhibition) of liquid-liquid fractions of the methanolic extract of *Vetiveria zizanioides* KS-1 on *M. smegmatis*.

	Mycobacterium smegmatis *strain Mc²155* (wld type)	*Mycobacterium smegmatis* strain Mc²155 (NalR) 6b	*Mycobacterium smegmatis* strain Mc²155 13a
Hex fraction	-	10 mm	4 mm
Ethyl acetate	-	-	-
Chloroform	-	5 mm	3 mm

- **The bioactivity testing against *Mycobacterium smegmatis* strain MC²155 showed that the hexane and chloroform fractions were inhibitory to the nalidixic acid resistant strains (NalR) (6b and 13a) but not to the wild type,**

- **This prompted us to carry out activity guided isolation and characterization of antimycobacterial agents from hexane extract of *V. zizanioides*.**

- *Vetiveria zizanioides* (L.) Nash (Poaceae) is popularly known as Vetiver or Khus grass.

- Its roots are the major source of well-known vetiver or Khus oil, which is used in medicine and in perfumery.

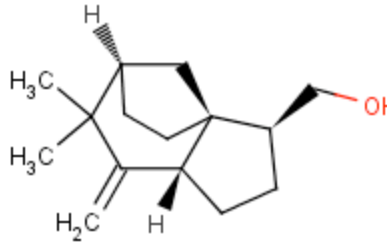
- Roots are stimulant, tonic, cooling, stomachic, diuretic, antispasmodic, and emmenagogue, and used in fevers, inflammations, and irritability of stomach.

- Various tribal people in the subcontinent use different parts of the grass for many of their ailments, such as boils, burns, epilepsy, fever, scorpion sting, snakebite, and sores in the mouth.

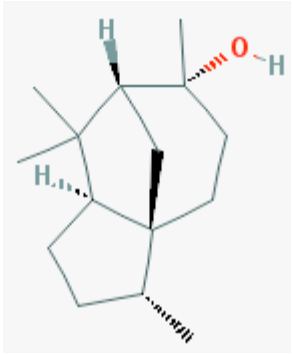
- The root paste is used for headache and toothache, the leaf paste is used for lumbago, sprain, and rheumatism, the stem decoction for urinary tract infection, the leaf juice as an anthelmintic, the vapors for malarial fever, and the root ash is given for acidity relief.

➢**Over 150 compounds have been isolated and characterized from vetiver oil mainly consisting of sesquiterpenes and their derivatives.**

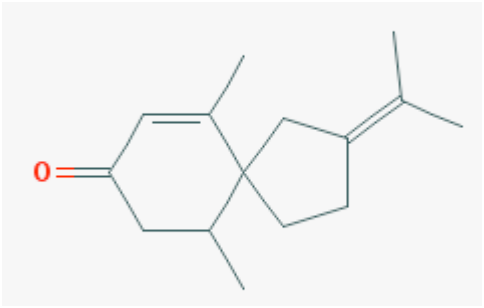
➢**Among these, the major active constituents identified are:**



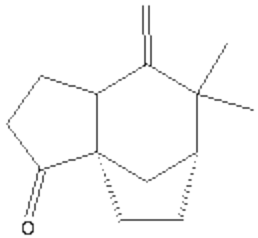
Khusimol



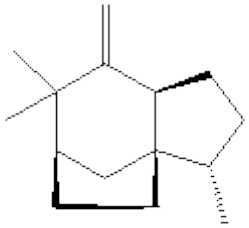
eudesmol



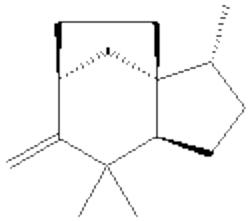
vetivone



khusimone



zizaene



prezizaene

➢**But the characteristic constituents are khusimol (3.4-13.7%), vetiselinenol (1.3- 7.8%), α-vetivone (2.5- 6.3%) and β-vetispirene (1.6-4.5%).**

The fresh roots of *V. zizanioides* (genotype KS 1 , *10 kg)*

- **To the best of our knowledge, no antimycobacterial compound has been identified from vetiver oil, hence for carrying out activity-guided fractionation, isolation, and characterization of antimycobacterial constituents from vetiver oil.**
- **The oil was obtained by steam distillation of roots.**

Isolation of vetiver oil

Steam Distt. → Oil in 1% yield (v/w) on a fresh weight basis

The freshly distilled vetiver oil (100 ml)

Subjected to CC

Gradient elution of column was carried out in increasing polarity Hexane-$CHCl_3$-MeoH

Vetiveria oil

- →Fr(s)1-54 eluted with Hexane (100%) → Fr(1-2) vz-1; Fr(5-7) vz-2; Fr(10-11) vz-3; Fr(17-24) vz-4; Fr(32-34) vz-5
- →Fr(s)55-81 eluted with Hex:$CHCl_3$ (95:5)
- →Fr(s)82-107 eluted with Hex:$CHCl_3$ (90:10)
- →Fr(s)108-139 eluted with Hex:$CHCl_3$ (85:15) → Fr(s) (110-119) vz-6
- →Fr(s)140-166 eluted with Hex:$CHCl_3$ (80:20)
- →Fr(s)167-187 eluted with Hex:$CHCl_3$ (75:25)
- →Fr(s)188-303 eluted with Hex:$CHCl_3$ (70:30) → Fr(s) (207-231) vz-7
- →Fr(s)304-336 eluted with Hex:$CHCl_3$ (65:35) → Fr(s) (298-324) vz-8
- →Fr(s)337-424 eluted with Hex:$CHCl_3$ (60:40) → Fr(s) (325-339) vz-9
- →Fr(s)425-474 eluted with Hex:$CHCl_3$ (55:45)
- →Fr(s)475-494 eluted with Hex:$CHCl_3$ (45:55)
- →Fr(s)495-506 eluted with Hex:$CHCl_3$ (40:60)
- →Fr(s)507-512 eluted with Hex:$CHCl_3$ (35:65)
- →Fr(s)513-518 eluted with Hex:$CHCl_3$ (30:70)
- →Fr(s)519-532 eluted with Hex:$CHCl_3$ (20:80)
- →Fr(s)533-559 eluted with $CHCl_3$ (100%)
- →Fr(s)560-570 eluted with MeOH:$CHCl_3$ (2:98)
- →Fr(s)571-578 eluted with MeOH:$CHCl_3$ (5:95)
- →Fr(s)579-584 eluted with MeOH:$CHCl_3$ (10:90)
- →Fr(s)585-589 eluted with MeOH (100%)

Table 1 List of *Mycobacterial* strains

S. no.	*M. smegmatis* strains	Specification
1	MC^2 155	Wild type (sensitive to quinolones and fluoroquinolones)
2	CSC-101	Resistance to ciprofloxacin, lomefloxacin, and norfloxacin
3	MDR-R	Resistance to rifampicin, tetracycline, and chloramphenicol
4	MDR-40	Resistance to rifampicin, ampicillin, and kanamycin
5	Lom R5	Resistance to lomefloxacin
6	MDRQ	Resistance to quinolones and ciprofloxacin

Vetiveria oil

→Fr(s)1-54 eluted with Hexane (100%) → →Fr(1-2) vz-1; →Fr(5-7) vz-2; →Fr(10-11) vz-3; →Fr(17-24) vz-4; →Fr(32-34) vz-5

→Fr(s)55-81 eluted with Hex:$CHCl_3$ (95:5)

→Fr(s)82-107 eluted with Hex:$CHCl_3$ (90:10)

→Fr(s)108-139 eluted with Hex:$CHCl_3$ (85:15) → Fr(s) (110-119) vz-6

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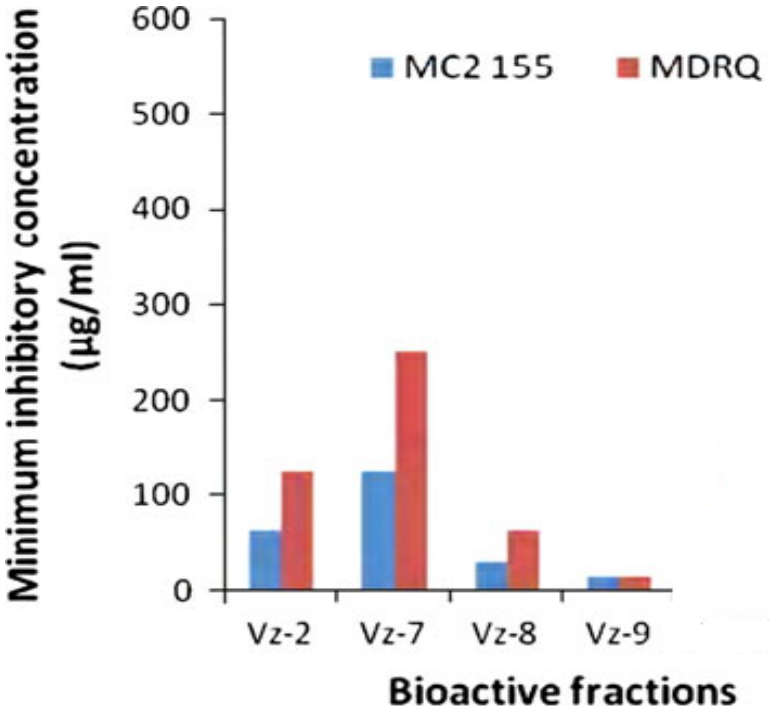
→Fr(s)304-336 eluted with Hex:$CHCl_3$ (65:35) → Fr(s) (298-324) vz-8

→Fr(s)337-424 eluted with Hex:$CHCl_3$ (60:40) → Fr(s) (325-339) vz-9



Four sub fractions Vz-2, Vz-7, Vz-8, and Vz-9 showed significant activity against wild-type (MC2155) and drug-resistant (MDRQ) strains of *M. smegmatis*

Table 2 MIC of bioactive fractions in µg/ml against *M. smegmatis* strains

MIC of bioactive fractions (µg/ml)	MC^2155	MDRQ
Root (intact)	125	125
Vz-1	>500	>500
Vz-2	**62.5**	125
Vz-3	500	500
Vz-4	500	500
Vz-5	500	500
Vz-6	500	500
Vz-7	125	250
Vz-8	**31.25**	**62.5**
Vz-9	**15.62**	**15.62**
Vz-7 M	**81.66**	163.33
Vz-8E	500	**62.5**
Ciprofloxacin	0.78	25
Lomofloxacin	0.78	>50
Nalidixic acid	50	50

Most active fraction

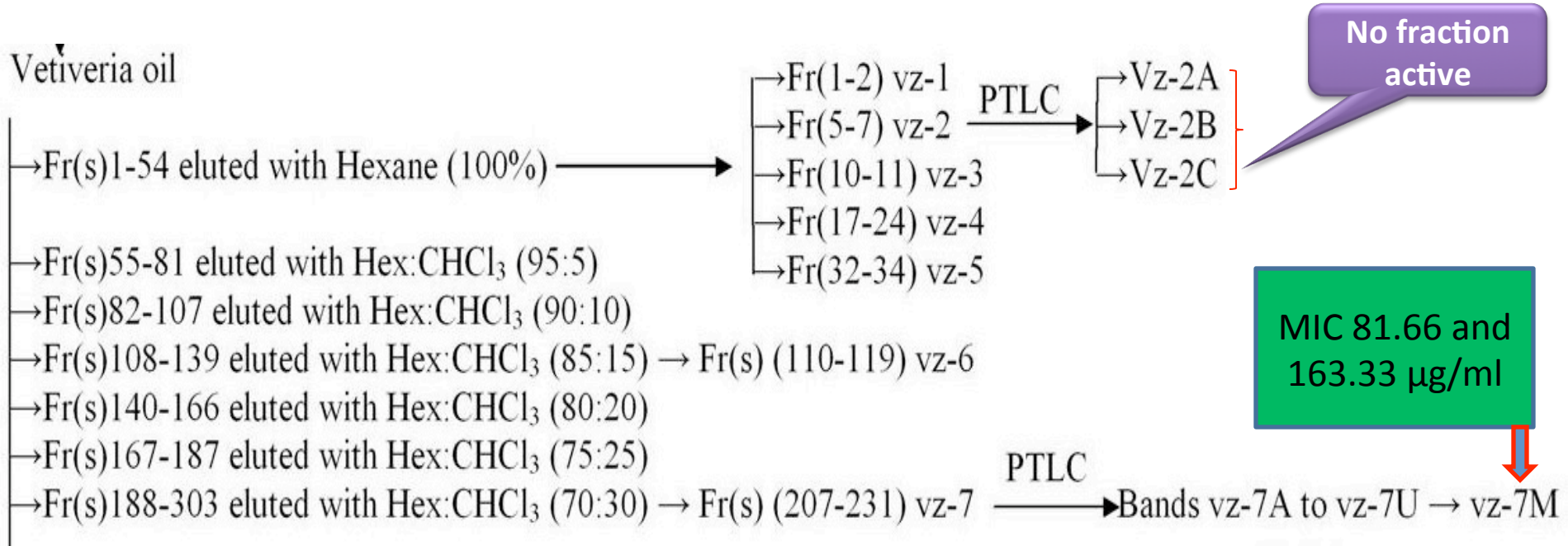
The bold value indicates the low MIC of fractions/compounds

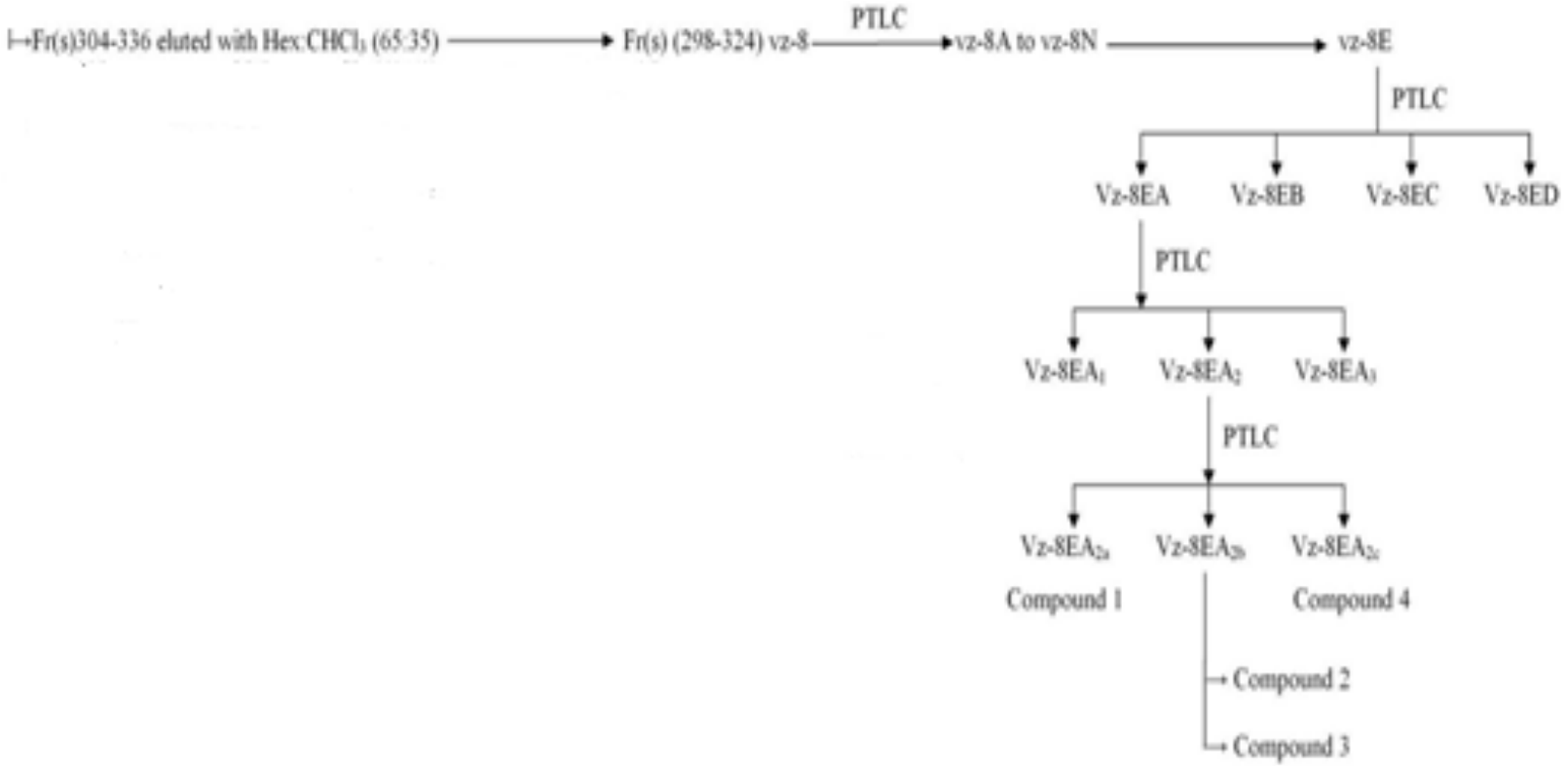
Isolation of antimycobacterial compounds from bioactive fractions

- For the isolation of bioactive molecules further purification of subfractions was carried out over PTLC. The sub fraction Vz-2 was purified into three fractions Vz-2A, Vz-2B, and Vz-2C.

- But none of the fractions were active. This demonstrates that the activity of sub fraction Vz-2 was due to cumulative effect of these isolated fractions.

- Similarly fraction Vz-7 on PTLC separation yielded twenty-one fractions (Vz-7A to Vz-7U), of which sub fraction Vz-7M showed moderate activity (MIC 81.66 and 163.33 µg/ml) against MC^2 155 and MDRQ strains of *M. smegmatis*, respectively

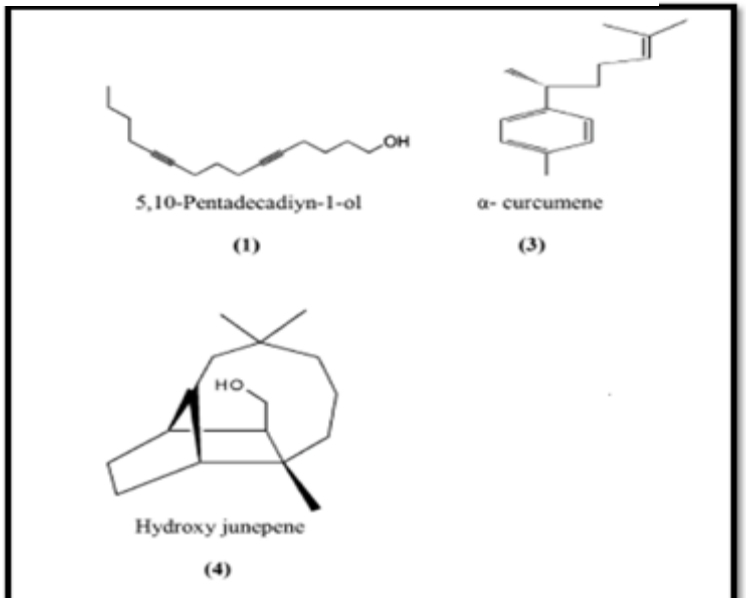




Further, fraction Vz-8 was subjected for repeated PTLC purification, which finally afforded three sub-fractions Vz-8EA2a, Vz-8EA2b, and Vz-8EA2c. The sub-fraction Vz-8EA2a was characterized as 5,10-pentadecadiyn-1-ol (1).

The sub-fraction Vz-8EA2b was a mixture of two compounds (2, 3), of which compound 2 with MW 220 could not be characterized while the other compound was characterized as α-curcumene (3).

The last sub-fraction Vz-8EA2c was characterized as hydroxy junipene (4).



→Fr(s)337-424 eluted with Hex:$CHCl_3$ (60:40) → Fr(s) (325-339) vz-9

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→Fr(s)571-578 eluted with MeOH:$CHCl_3$ (5:95)

→Fr(s)579-584 eluted with MeOH:$CHCl_3$ (10:90)

→Fr(s)585-589 eluted with MeOH (100%)

PTLC

vz-9A to vz-9J → vz-9A

PTLC

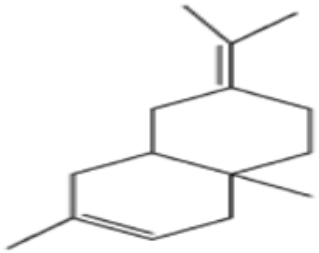
$Vz\text{-}9A_1$ $Vz\text{-}9A_2$ $Vz\text{-}9A_3$

Unknown Unknown

→Compound 5

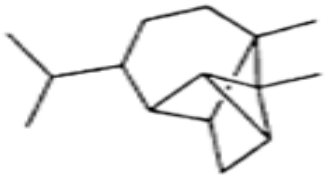
→ Compound 6

→ Compound 7



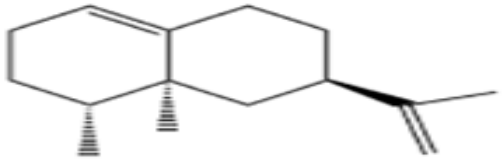
Selina-3,7(11)-diene

(5)



(+) cycloisosativene

(6)



Valencene

(7)

All the isolated compounds were active against the one sensitive and five resistant strains of M. smegmatis.

Compound (1) was most active against both the drug-resistant strains, MDR-R (resistant to tetracycline, chloramphenicol, and rifampicin) and MDR-40 (ampicillin, kanamycin, and rifampicin) resistant with MIC 31.25 µg/ml also active (MIC 62.5 µg/ml) against CSC-101 (resistant to ciprofloxacin, lomefloxacin, and norfloxacin)

Compounds 2, 3 & 4 were equally active (MIC 62.5 µg/ml) against MC² 155 (sensitive to quinolones and fluoroquinolones) strain

compound 5, 6, 7 were more active against MDR-R and MDR-40 resistant strains of *M. smegmatis*.

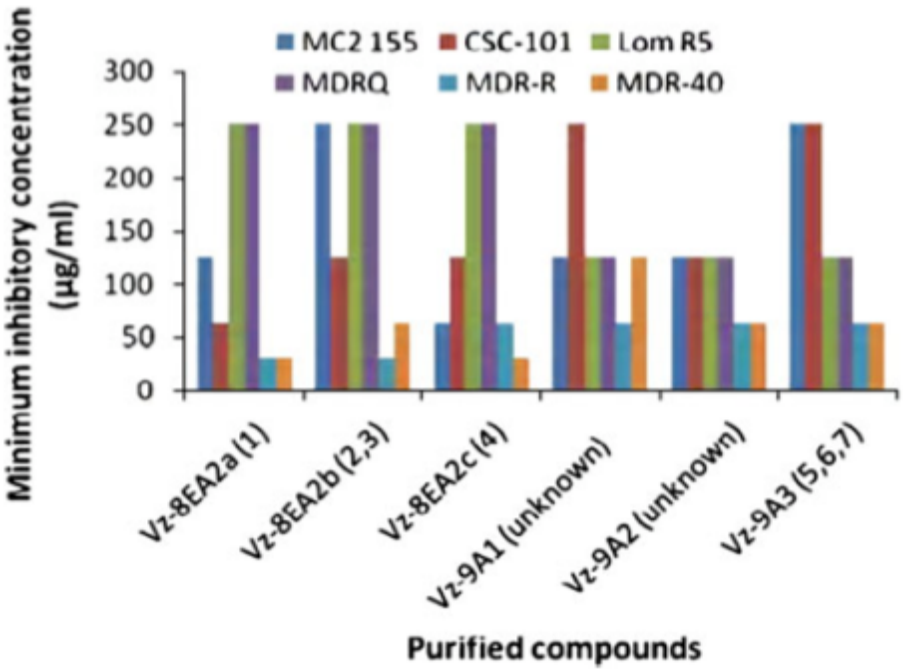


Fig. 3 Antimycobacterial potential of purified compounds

Table 3 MIC of purified compounds in µg/ml against *M. smegmatis* strains

MIC of bioactive fractions and purified compounds (µg/ml)	MC²155	CSC-101	Lom R5	MDRQ	MDR-R	MDR-40
$Vz\text{-}8EA_{2a}$ (1)	125	**62.5**	>250	250	**31.25**	**31.25**
$Vz\text{-}8EA_{2b}$ (2, 3)	250	125	>250	>250	**31.25**	**62.5**
$Vz\text{-}8EA_{2c}$ (4)	**62.5**	125	>250	>250	**62.5**	**31.25**
$Vz\text{-}9A_1$ (unknown)	125	250	125	125	**62.5**	125
$Vz\text{-}9A_2$ (unknown)	125	125	125	125	**62.5**	**62.5**
$Vz\text{-}9A_3$ (5, 6, 7)	250	250	125	125	**62.5**	**62.5**
Ciprofloxacin	0.78	3.125	25	25	3.125	>25
Lomofloxacin	0.78	<01	>50	>50	10	2.5
Nalidixic acid	0.78	6.25	25	50	50	50

The bold value indicates the low MIC of fractions/compounds

From the bioactivity profile of fraction Vz-9 (Table 2) and its PTLC fractions Vz-9A1, Vz-9A2, and Vz-9A3 (Table 3), it is evident that the most potential activity of the original fraction Vz-9 was due to cumulative effect of isolated constituents which got distributed in fractions on further separation over PTLC .

Table 2 MIC of bioactive fractions in µg/ml against *M. smegmatis* strains

MIC of bioactive fractions (µg/ml)	MC^2155	MDRQ
Root (intact)	125	125
Vz-1	>500	>500
Vz-2	**62.5**	125
Vz-3	500	500
Vz-4	500	500
Vz-5	500	500
Vz-6	500	500
Vz-7	125	250
Vz-8	**31.25**	**62.5**
Vz-9	**15.62**	**15.62**

Table 3 MIC of purified compounds in µg/ml against *M. smegmatis* strains

MIC of bioactive fractions and purified compounds (µg/ml)	MC^2155	CSC-101	Lom R5	MDRQ	MDR-R	MDR-40
$Vz\text{-}8EA_{2a}$ (**1**)	125	**62.5**	>250	250	**31.25**	**31.25**
$Vz\text{-}8EA_{2b}$ (**2**, **3**)	250	125	>250	>250	**31.25**	**62.5**
$Vz\text{-}8EA_{2c}$ (**4**)	**62.5**	125	>250	>250	**62.5**	**31.25**
$Vz\text{-}9A_1$ (unknown)	125	250	125	125	**62.5**	125
$Vz\text{-}9A_2$ (unknown)	125	125	125	125	**62.5**	**62.5**
$Vz\text{-}9A_3$ (**5**, **6**, **7**)	250	250	125	125	**62.5**	**62.5**
Ciprofloxacin	0.78	3.125	25	25	3.125	>25
Lomofloxacin	0.78	<01	>50	>50	10	2.5
Nalidixic acid	0.78	6.25	25	50	50	50

Conclusions

- **Our studies provided evidence that vetiver root oil, its fractions and isolated compounds possess significant antimycobacterial activity against the drug-resistant strains of *M. smegmatis.***

- **The antimycobacterial activity presented here is being reported for the first time in the fractions and compounds isolated from the vetiver root oil.**

- **However, further studies are needed to confirm the corresponding mechanisms of action.**

- **These results may be of great help in antimycobacterial drug development from a very common, inexpensive, and non toxic natural product.**

- **Our results also encourage for Structure Activity Relationship "SAR" study of some of the isolated molecules.**

Shikha Gupta

Gaurav R. Dwivedi

M. P. Darokar

THANK YOU