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In Vitro Activity of Selected Medicinal Plant Extracts Against *Mycobacterium tuberculosis* and other Non Mycobacterial Pathogens

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ABSTRACT

Use of medicinal plants for the treatment of infectious and life style diseases is since time immemorial. This study reports the in vitro antibacterial activity of selected medicinal plant extracts against *Mycobacterium tuberculosis* H37Rv and other non-mycobacterial pathogens. Fruits of *S. torvum, Z. mauritiana* and leaves of *V.negundo* were sequentially extracted using n-hexane, ethyl acetate and methanol. All n-hexane, ethyl acetate and methanol extracts were screened against clinical pathogens viz., *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, Carbapenem resistant *K. pneumoniae* ATCC 700603 and *V. parahaemolyticus* by agar well diffusion method at 10 µg/µL concentration. The methanol extract of S. torvum and ethyl acetate extract of *V.negundo* and *Z.mauritiana* demonstrated 17 and 23 mm inhibition against the non-mycobacterial pathogens, respectively. The three extracts were also screened for anti-tubercular activity against *M. tuberculosis* H37Rv using Luciferase Reporter Phage (LRP) assay. All the three extracts were exhibited anti TB activity at 500 µg/ml concentration. In particular, the *S. torvum* extract was showed 98.46% inhibition. GC-MS analysis of the afore mentioned extracts yielded peaks of compounds of ethnomedicinal value/significance. Findings of this study depicted that the medicinal plant *S. torvum* deserves the potential for isolation of anti TB molecules.

KEY WORDS: ANTI-TB ACTIVITY, MEDICINAL PLANTS, MYCOBACTERIUM TUBERCULOSIS, TUBERCULOSIS.

INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains among the top ten mortality causing diseases in the world. According to WHO, 10 million new cases of TB and 1.3 million deaths were estimated globally in 2017. This puts the disease burden of TB to be equivalent to 133 deaths per 1,00,000 people. Around 27% of the global TB cases were contributed by India in 2017 which highlights the burden of the disease on the Indian population. The severity of multi-drug resistance TB still persists and the success rates of treatment for MDR/RR-TB (Multi-drug resistant/Rifampicin resistant TB) and XDR-TB (Extensively drug resistant TB) are strikingly low, being 55% and 34% respectively. The problem of drug resistance does not appear to have an easy solution in the near future. Synthetic drugs used in the TB treatment programme pose a heavy burden on the already weakened body of TB patients, (WHO Global TB report 2019).

The drugs are most commonly nephrotoxic (Hussein et al., 2015) and hepatotoxic (Ramappa et al., 2012), thereby leading to harmful side effects of the treatment. This shifts

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the focus of the treatment regime to natural products and medicinal herbs for treatment of infectious diseases. Use of ethno-medically significant plants for treatment can be initiated by validating the traditional methods that were reported to be used since ancient times. These methods were used to treat multiple ailments including bacterial and viral diseases. With several reports on the activity of herbal medicines against pathogens, there is a need to focus on the potential of herbal medicines in the treatment of infectious diseases,(Ladda and Magdum 2018).

Solanum torvum is a shrub of the Solanaceae family widely available and extensively used in India for the treatment of bacterial diseases and for relief from cough and cold (Yousaf et al., 2013). It is native to other tropical countries and has been traditionally used for treatment of various ailments such as a sedative and diuretic, TB, skin infections, fever and tooth decay (Naimon et al., 2015; Silva et al., 2011). The plant extracts have been reported to be possess cardioprotective, nephroprotective, anti-viral, anti-microbial, anti-oxidant, anti-ulcerogenic and haemostatic properties (Jaiswal et al., 2012). Such properties could be attributed to the presence of phytoconstituents such as flavonoids, steroids, saponins, tannins, alkaloids, vitamin B, vitamin C and phenols, (Jaiswal et al., 2012; Yousaf et al. 2013 Ladda and Magdum (2018). *Ziziphus mauritiana* is a member of the Rhamnaceae family and is native to the Indian subcontinent, Africa, Iran and parts of Southern Asia. It is known to be traditionally used for the treatment of pain, vomiting and diarrhoea (Mahesh et al., 2008). It has also been reported to possess anti-plasmodial effect (Sameera et al., 2015); its seeds are good sedatives while its leaves are used for treatment of sores, cuts and ulcers. It has also been demonstrated to resolve liver troubles, asthma and experimentally induced liver damage (Abalaka et al., 2010). The juice obtained from the bark of its root is known to alleviate gout and rheumatism according to traditional medicinal practises (Priyanka et al., 2015).

It is a pharmacologically important plant since it is known to possess anti-typhoid, anti-cancer, antioxidant and anti-inflammatory properties (Abdallah et al., 2016). *Vitex negundo* is a member of the *Verbenaceae* family and is native to South Asia, East Africa, South America, Indonesia and Japan. It has been demonstrated to possess analgesic, anti-oxidant, anti-inflammatory, hypoglycemic, anti-tumour, anti-rheumatism and insecticidal activities (Zheng et al., 2015, Gupta et al., 2010). The leaves of the plant have been proven to possess anti-convulsant and anti-parasitic activities (Ladda and Magdum 2018) which is an addition to the traditional medicinal properties that the plant has been reported to possess. In this study, we have investigated the antibacterial and anti-mycobacterial properties of different solvent extracts of *S. torvum* fruits, *Z.mauritiana* fruits and leaves of *V.negundo*. GC-MS analysis was performed to identify the potential compounds present in the extracts. With the help of the present in vitro studies, it may be possible to maximize the traditional use of the plants for treatment of various infections.

MATERIALS AND METHODS

The fruits of Solanum torvum, fruits of *Ziziphus mauritiana* and leaves of *Vitex negundo* were collected in 2018, from Chennai, India, and duly authenticated by a botanist. The fruits and leaves were processed by washing thrice with distilled water and surface sterilization by rinsing with 70% acetone. Then the samples were shade dried and powdered using mixer grinder. Sequential extraction was performed using the solvents n-hexane, ethyl acetate and methanol to extract the compounds from the powdered plant material. Ten gram of powdered plant material was added to 100 mL n-hexane and incubated at room temperature in an orbital shaker at 80 rpm for 24 hrs. The extract was filtered using Whatman filter paper and the powdered plant material was reused upon drying. To the dried powder, 100 mL of ethyl acetate and subsequently methanol were added and the extracts were filtered and collected in a similar manner. The n-hexane, ethyl acetate and methanol extracts were concentrated using rotary evaporator and dried by incubating the extracts at 50 ^{o}C for 24-36 hrs. The weight of the extracts was determined and the extracts were dissolved in 10% DMSO for evaluating their antibacterial and anti-mycobacterial activities. Anti-bacterial activity of the extracts was evaluated by agar well diffusion method. Overnight grown cultures of bacterial pathogens viz. *Staphylococcus aureus* ATCC 29213, Pseudomonas aeruginosa ATCC 27853, Carbapenem resistant *Klebsiella pneumoniae* ATCC 700603 and *Vibrio parahemolyticus* were swabbed on individual Muller-Hinton Agar plates. Wells of 5 mm diameter were cut on the plate using well cutter and 10 µg/µL of n-hexane (HF), ethyl acetate (EAF) and methanol (MF) extracts were added to each well respectively.

The plates were then incubated at 37 ^{o}C for 18 hours and zones of inhibition were measured. The assay was performed in triplicates and mean values were calculated and tabulated. The antitubercular activity of HF, EAF and MF was screened against M. tuberculosis H37Rv by Luciferase Reporter Phage assay (Radhakrishnan et al., 2016). Briefly, 400 µL of middlebrook 7H9 broth was added to two cryovials (Control) and another 400 µL of rifampicin containing Middlebrook 7H9 broth at concentration of 2 µg/mL was added to a cryovial (drug control). About 350 µL of Middlebrook 7H9 was added to two cryovials (solvent control and test). Fifty µL of extract (HF) was added to test vial to achieve a final concentration of 500 µg/mL and 50 µL of 10% DMSO was added to solvent control vial. All the vials were added with 100 µL of M. tuberculosis H37Rv (McFarland unit 2) and incubated at 37 ^{o}C for 72 hrs. After incubation, 50 µL of phAE202 and 40 µL of 0.1 M CaCl2 were added to all the vials (Cell-phage mixture) and incubated at 37 ^{o}C for 4 hrs. About 100 µL of cell-phage mixture from each vial was added into a luminometer cuvette, to which 100 µL of D- Luciferin was added. The relative light unit

(RLU) was measured immediately at 10 sec integration time using the luminometer (Lumat 9508, Berthold, Germany). The above mentioned procedure were followed for the other extracts MF and EAF as well. The percentage reduction was calculated using the formula:% RLU reduction = Control RLU- Test RLU/ Control RLU x 100 On comparison with control, if the sample showed 50% or more reduction of RLU, the extract was deemed to have anti tubercular activity. The potential MF extract was analysed by GC-MS (Shimadzu QP2010 Ultra).

RESULTS AND DISCUSSION

Sequential extraction procedures are commonly used to isolate a number of compounds from plant extracts. The process offers improved phase-specificity due to combined use of multiple solvents of varying polarity. In sequential extraction, the plant residue from the first extraction is used as the material for the second extraction and the process may be continued as required (Kaplan et al., 2009). In this study, sequential extraction was carried out using solvents of increasing polarity. Due to the difference in chemical nature of the solvents, the process is very selective in extraction of compounds from plants. Sequential extraction from 10 g of plant extracts using 100 mL of solvents n-hexane, ethyl acetate and methanol yielded fractions whose stock concentrations were maintained at 10 mg/mL. The crude extracts were diluted and a final concentration of 10 µg/µL was used for evaluating anti-microbial activity against the selected non-mycobacterial pathogens. The inhibitory activity of n-hexane, ethyl acetate and methanol extracts of the fruits of *S. torvum,* fruits of *Z.mauritiana* and leaves of *V.negundo* were screened against clinical pathogens and results were summarized in Table 1.

The methanol extract (MF) of *S.toruvum* fruits demonstrated inhibitory activity against *S. aureus* ATCC 29213 (17mm), *P. aeruginosa* ATCC 27853 (17mm) and *V. parahaemolyticus* (25mm) at a concentration of 10 µg/µL whereas the ethyl acetate extract (EAF) showed inhibition against *P. aeruginosa* ATCC 27853 (12mm) and V. parahaemolyticus (12mm) at the same concentration. None of the pathogens tested were inhibited by hexane extract (HF). Chah et al. (2000) demonstrated the antimicrobial activity of methanolic and ethanolic extracts of S. torvum against Actinomyces pyogenes, B. subtilis, S.pyogenes, A.niger and *C.albicans*. In other reports, plant extracts of *S. torvum* showed inhibition against *B.cereus, Staphylococcus epidermidis*, *E. coli, V. cholerae, Salmonella cibrum* and *Salmonella typhimurium* (Naimon et al., 2015; Sivapriya et al., 2011).

The ethyl acetate extract (EAF) of *Z.mauritiana* fruits inhibited the growth of *S. aureus* ATCC 29213 (23mm), *P.aeruginosa* ATCC 27853 (11mm) and Carbapenem resistant *K.pneumoniae* ATCC 700603 (11mm) at a concentration of 10 µg/µL. However, 10 µg/µL of MF inhibited the growth of only *S.aureus* ATCC 29213 (12mm) and *V.parahaemolyticus* (12mm), while the HF did not inhibit the growth of any of the pathogens tested. The antibacterial activity of the extracts demonstrated against *S.aureus* is in accordance with previous reports of the same being exhibited by extracts of *Z.mauritiana* as reported by Mahesh et al., 2008, Abdallah et al., 2016 and Priyanka et al., 2015. Their reports also illustrated the anti-bacterial activity of *Z.mauritiana* extracts against bacterial pathogens such as *B.subtilis, E.coli*, *Pseudomonas fluorescens* and *K.pneumoniae*.

EAF of *V.negundo* leaves was inhibited the growth of *S.aureus* ATCC 29213 (15mm) and *P.aeruginosa* ATCC

Table 1. Activity of extracts tested against non-mycobacterial pathogens

S. No.	Plant	Extract	Zone of inhibition against pathogen (mm in diameter)			
			S. aureus ATCC 29213	*P. aeruginosa* ATCC 27853	Carbapenem resistant *K. pneumoniae* ATCC 700603	*Vibrio parahem-olyticus*
1	*Solanum torvum*					
	HF	-	-	-	-	
		EAF	-	12	-	12
		MF	17	17	-	25
2	*Ziziphus mauritiana*					
	HF	-	-	-	-	
		EAF	23	11	11	-
		MF	12	-	-	12
3	*Vitex negundo*					
	HF	-	-	-	25	
		EAF	15	15	-	-
		MF	-	-	-	-

27853 (15mm) at a concentration of 10 µg/µL. The HF only inhibited the growth of *V. parahaemolyticus* (25 mm), while the MF did not inhibit the growth of any of the pathogens tested. This was similar to the reports of antibacterial activity of *V.negundo* extracts against *S.aureus*, *V.parahaemolyticus* and *K.pneumoniae* (Zheng et al., 2015). This helps to substantiate the ethnomedicinal use of *V.negundo* for the treatment of cold, cough and bacterial dysentery (Gupta et al., 2010).

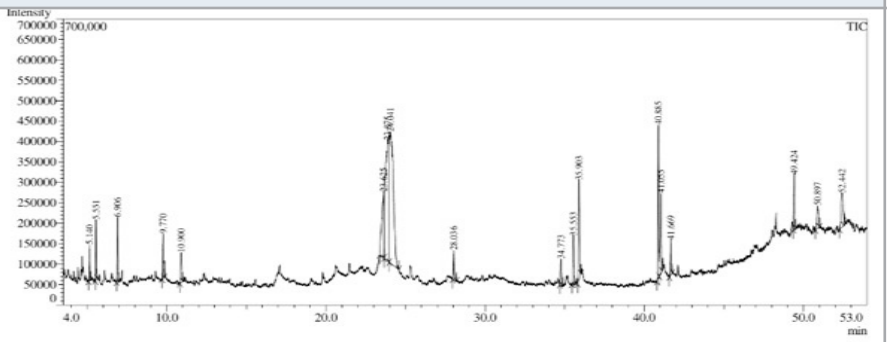
Table 2. Anti-tubercular activity of extracts against *M. tuberculosis* H37Rv by LRP

Extracts	% inhibition against *M. tuberculosis* H37Rv
S.torvum-MF	98.46
Z.mauritiana-EAF	84.41
V.negundo-EAF	59.2

Upon screening of the extracts against *M. tuberculosis* H37Rv by Luciferase Reporter Phage (LRP) assay, MF of *S.torvum* showed promising inhibitory activity against *M. tuberculosis* H37Rv at 500 µg/mL concentration and the RLU reduction in terms of inhibition was found to be 98.46% (Table 2). Our results are in correlation with that of Mohamad et al., (2011), in which hydromethanolic fruit extracts from *S.torvum* displayed moderate antimycobacterial activity against *M. tuberculosis*

Figure 1: (a) GC-MS chromatogram (b) GC-MS Peak Report of Methanol fraction of *S. torvum*

a



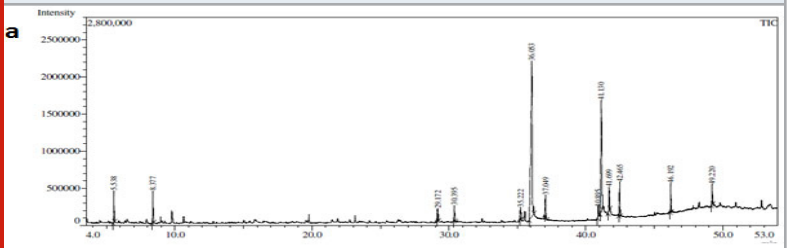
b

Peak Report TIC

Peak#	R.Time	Area	Area%	Name
1	5.140	259030	1.30	4-Methylpiperidine-1-carboxylic acid, pheny
2	5.551	392052	1.96	1,2,3-Propanetriol, 1-acetate
3	6.906	447519	2.24	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-
4	9.770	424886	2.13	1,2,3-Propanetriol, 1-acetate
5	10.900	321485	1.61	.beta.-Alanine, N-acryloyl-, isobutyl ester
6	23.625	1520222	7.61	1,2-CYCLOPROPANEDIACETIC ACID
7	23.875	4950139	24.79	1,3,4,5-TETRAHYDROXYCYCLOHEXAN
8	24.041	5546944	27.78	Quinic acid
9	28.036	335246	1.68	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxypl
10	34.773	288487	1.44	Benzenepropanoic acid, 3,5-bis(1,1-dimethyl
11	35.553	457019	2.29	Dibutyl phthalate
12	35.903	946639	4.74	n-Hexadecanoic acid
13	40.885	1379261	6.91	9,12-Octadecadienoic acid (Z,Z)-
14	41.055	885544	4.44	Oleic Acid
15	41.669	312407	1.56	Octadecanoic acid
16	49.424	374181	1.87	Pyrazolo[3,4-b]thiopyrano[4,3-d]pyridin-1-a
17	50.897	379027	1.90	STIGMAST-5-EN-3-OL, (3.BETA.)-
18	52.442	746291	3.74	Hexacontane
		19966379	100.00	

Figure 2: (a) GC-MS chromatogram (b) GC-MS Peak Report of Ethyl acetate fraction of *Z.mauritiana*.

a



b

Peak Report TIC

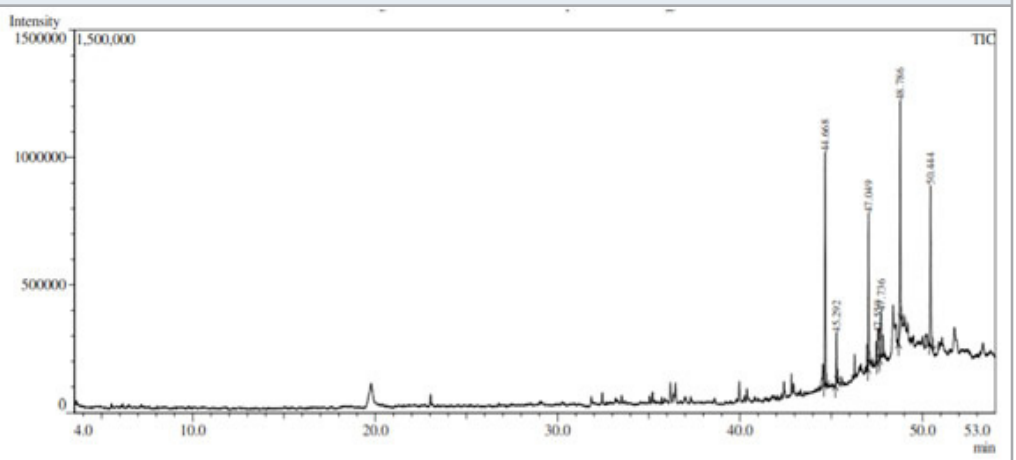
Peak#	R.Time	Area	Area%	Name
1	5.538	1006609	3.31	1,2,3-Propanetriol, 1-acetate
2	8.377	1196836	3.93	4-(1-HYDROXY-ETHYL) .GAMMA. BUT
3	29.172	507078	1.67	TETRADECANOIC ACID
4	30.395	681681	2.24	1-Nonadecene
5	35.222	584657	1.92	Palmitoleic acid
6	36.053	14232713	46.79	l-(+)-Ascorbic acid 2,6-dihexadecanoate
7	37.049	1051697	3.46	1-Nonadecene
8	40.895	489123	1.61	9,12-Octadecadienoic acid (Z,Z)-
9	41.130	6818319	22.42	Oleic Acid
10	41.699	942149	3.10	Octadecanoic acid
11	42.465	1235942	4.06	1-Heptacosanol
12	46.192	983341	3.23	Heneicosyl trifluoroacetate
13	49.220	687762	2.26	1-Triacontanol
		30417907	100.00	

H37Rv.

Previously other parts of plants are also evaluated for antimycobacterial activity includes the ethanol extract of leaves from *S. torvum,* which displayed activity against *M. smegmatis* and *M. tuberculosis* H37Rv (Nguta et al., 2016). In this study, *Z.mauritiana* EAF was showed 84.41% inhibition against *M.tuberculosis* H37Rv at a concentration of 500 µg/mL. Using the alamar plate assay, *Z.mauritiana* was reported to have anti-mycobacterial activity against *M.tuberculosis* H37Ra as reported by Panseeta et al., (2011). *V.negundo* EAF demonstrated an anti-mycobacterial activity of 59.2% at a concentration of 500 µg/mL. This is along the lines of the published reports of Ladda et al., (2018) and Gupta et al., (2010) where the plant extracts were tested against *M. tuberculosis* H37Rv. GC-MS analysis of MF of *S.torvum* (Fig. 1) showed several compounds among which the prominent peaks denotes the presence of Quinic acid, n-hexadecanoic acid, oleic acid and 9,12-octadecadienoic acid. The GC-MS peaks of EAF of *Z. mauritiana* (Fig. 2) yielded significant peaks corresponding to ascorbic acid-2,6-dihexadecanoate and oleic acid, while those of *V.negundo* yielded peaks (Fig. 3) which indicated the presence of 2-heptanone, 4,8,13-cyclodecatriene and 2-pentanoic acid. The antimicrobial and anti-mycobacterial activity of the extracts could be attributed to the presence of oleic acid as reported by Ojo et al., (2018; Kalita et al., 2018 and Santhosh et al., 2013).

Fatty acids such as n-hexadecanoic acid, otherwise known as palmitic acid has also been reported to possess anti-mycobacterial activity (Ojo et al., 2018). Ascorbic acid, 2,6-dihexadecanoate is an antioxidant which could play a significant role in combatting deficiency or imbalance of essential nutrients which is a common problem that occurs in patients infected by TB. The ascorbic acid from the extracts could compensate for the decreased anti-oxidant levels and further elevate and complement the effects of the treatment (Turchenko et al., 2008).

Figure 3: (a) GC-MS chromatogram (b) GC-MS Peak Report of Ethyl acetate fraction of *V.negundo*.



b

Peak Report TIC

Peak#	R.Time	Area	Area%	Name
1	44.668	2733406	22.73	2-HEPTANONE, 6-(3-ACETYL-1-CYCLO
2	45.292	564349	4.69	Sclareoloxide(Cis-A/B)
3	47.049	1717107	14.28	TETRAHYDROEDULAN D
4	47.550	842980	7.01	Octadecane, 3-ethyl-5-(2-ethylbutyl)-
5	47.736	1083374	9.01	Tetrapentacontane
6	48.786	2821424	23.46	4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-tr
7	50.444	2261534	18.81	2-PENTENOIC ACID, 5-(DECAHYDRO-5
		12024174	100.00	

CONCLUSION

In conclusion, *S. torvum* fruits, fruits of *Z.mauritiana* and leaves of *V.negundo* have potential anti-mycobacterial properties which can be taken up for further in vitro and in vivo studies. Phyto constituents of the plant extracts can be purified and investigated further to identify lead compounds responsible for anti-bacterial and anti-mycobacterial activity.

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Conflict of Int7erest: There are no conflict of Interest

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