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Anti-mycobacterial activity of *Acacia nilotica* leaves extracts against *Mycobacterium smegmatis*

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Abstract
The present investigation was carried out to determine the anti-mycobacterial activity of hexane, chloroform, butanol, ethanol, aqueous extracts and active acetone fraction of *Acacia nilotica* leaves by disc diffusion, minimum inhibitory concentration, and minimum bactericidal concentration tests against *Mycobacterium smegmatis*. In disc diffusion method, the active acetone fraction of *A. nilotica* leaves showed best inhibiting activity compared to other extracts. It showed diameter of 52 mm zone of inhibition at 150 mg/ml concentration. Whereas the standard dapsone showed 67 mm zone of inhibition at 50 mg/ml. In MIC test, among other extracts, the active acetone extract showed lowest MIC at 0.15 mg/ml. In MBC test also the active acetone extract comparatively showed minimal MBC at 0.28 mg/ml.

Keywords: *Acacia nilotica*, anti-mycobacterial activity, *Mycobacterium smegmatis*, disc diffusion, MIC, MBC

Introduction
Plants represent an important source of drugs, considering the wide diversity of molecules with medicinal potential, and can make an effective contribution to the search of new bioactive products, semi-synthetic medicines or lead compounds for the synthesis of medicines. The exploitation of this potential medicine source requires the bringing together of ethnobotanical, ethnopharmacological, chemical, biological, pharmacological and toxicological studies [1, 2].

Previous antimicrobial studies of *Acacia nilotica* proved it to be a good antibacterial agent. The crude extract of the leaves of the plant showed *In vitro* antiviral activity against the Turnip mosaic virus [3]. *Acacia nilotica* showed highest antibacterial activity against *Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Proteus mirabilis, Salmonella paratyphi B, Klebsiella pneumonia* [4]. Extract of *Acacia nilotica* leaves have *In vitro* antiplasmodial activity against *Plasmodium falciparum 3D* (chloroquine sensitive) and *Dd2* (chloroquine resistant and pyrimethamine sensitive) organisms [5]. In spite of these antibacterial studies there was a report that ancient people have used *Acacia nilotica* as a treatment for Leprosy. Thus the plant *Acacia nilotica* has been chosen to study the antimycobacterial activity against leprosy.

Leprosy remains a major public health problem, since single and multi-drug resistance has been reported worldwide over the last two decades. Leprosy was originated long back, over 5000 years ago, going back to the Neolithic times [6]. Leprosy is an infectious disease caused by a bacillus, *Mycobacterium leprae*. *M. leprae* multiplies slowly and the incubation period of the disease, on average, is 5 years. Symptoms may occur within 1 year but can also take as long as 20 years or even more to occur. The disease mainly affects the skin, the peripheral nerves, mucosa of the upper respiratory tract, and the eyes. Untreated, leprosy can cause progressive and permanent damage to the skin, nerves, limbs, and eyes. There were 208619 new leprosy cases registered globally in 2018, according to official figures from 159 countries from the 6 WHO Regions.

Mycobacterium smegmatis is an acid-fast bacterial species in the phylum Actinobacteria and the genus *Mycobacterium*. It is 3.0 to 5.0 µm long with a bacillus shape. *M. smegmatis* is useful for the research analysis of other Mycobacteria species in laboratory experiments. *M. smegmatis* is commonly used in work on the mycobacterium species due to its being a "fast grower" and non-pathogenic. *M. smegmatis* is a simple model that is easy to work with, i.e., with a fast doubling time and only requires a biosafety level 1 laboratory. The time and heavy infrastructure needed to work with pathogenic species prompted researchers to use *M. smegmatis* as a model for mycobacterial species. This species shares more than 2000 homologs with *M. tuberculosis* and shares the same unusual cell wall structure of *M. leprae*

and other mycobacterial species. There are 1046 identified orthologous proteins common to the two networks of *M. leprae* and *M. smegmatis*. Furthermore, it is readily cultivable in most synthetic or complex laboratory media, where it can form visible colonies in 3–5 days. These properties make it a very attractive model organism for other non-cultivable mycobacterial pathogens [7].

Thus the present study is an initiative to investigate the antileprosy property of *Acacia nilotica*, the anti-mycobacterial activity of *Acacia nilotica* is studied with *Mycobacterium smegmatis*.

Materials and Methods

Sample collection and identification

The study plant *Acacia nilotica* was collected from T.M Palayam (Latitude 10.9º N and Longitude 76.9º E) of Coimbatore in Tamilnadu. The collected plant sample was identified at Botanical Survey of India, Southern regional Centre, Coimbatore. The sample was stored in shadow places for further analysis.

Preparation of extracts

The shadow dried *A. nilotica* sample was powdered with help of electronic blender. Twenty five gram of powdered plant material was taken in clean sterile Soxhlet apparatus and extraction was done with 250 ml of different solvents (low polar to high polar) like as hexane, chloroform, butanol, ethanol and water. Similarly, another 10 grams of *A. nilotica* powder was extracted with 100 ml of acetone. After extraction the extracts were dried in room temperature until extract reach into solid form. From the solid extract suitable concentrations were made using Dimethyl sulfo-oxide (DMSO) for further analysis.

Further antimicrobial activity was performed for each extract was studied and found that acetone extract has maximum antimicrobial activity against the organisms used, so acetone extract is further purified by thin layer and column chromatography to obtain active acetone fraction in an purified form which is used for further analysis in antimycobacterial analysis along with other extracts.

Pathogenic strains

In the present study, the human pathogenic bacterial strain *Mycobacterim smegmatis* were obtained from Clinical laboratory in Valasaravakkam, Chennai, Tamil Nadu, India, and maintained on Trypton Soy Agar plates at 37 ºC.

Disc diffusion method

The antimicrobial potential of plant extracts against *Mycobacterium smegmatis* culture was examined by Disk Diffusion method [8]. *Mycobacterium smegmatis* culture was inoculated in 5ml Mueller-Hilton Broth incubated at 37 ºC for two hours. After the incubation, microbial lawn was prepared by pouring 100 µl of culture on the Mueller-Hilton Agar plates. Disks soaked with various concentrations of study plant extracts and standard Dapsone were placed onto the lawn and plates were incubated at 37 ºC for 24-48 hours. Antimicrobial potential of the plant extracts were evaluated on the basis of appearance and size of the zone of inhibition.

Minimum inhibitory concentration test

Minimum inhibitory concentration (MIC) of the plant extracts were determined by Micro Broth Dilution method using 96-well microtitre plate [9]. Briefly, *Mycobacterium smegmatis* culture was inoculated in 5ml Mueller-Hilton Broth incubated at 37 ºC for two hours. The various extracts of *A. nilotica* were diluted to obtain concentration ranging from 100 µg - 500µg /ml. serial dilutions of plant extracts were made in 100 µl Mueller-Hilton broth. Subsequently, 10 µl of two hour old culture was added to each well. One well served as a medium control while other served as culture control. The plates were incubated for 24 hours at 37 ºC. The MIC was considered as the lowest concentration of the well that exhibited reduction of bacterial colony as measured from turbidity of culture by optical density value.

Minimum bactericidal concentration test

Minimum Bactericidal concentration (MIC) of the plant extracts were determined by Micro Broth Dilution method using 96-well microtitre plate [9]. *Mycobacterium smegmatis* culture was inoculated in 5ml Mueller-Hilton Broth at incubated at 37 ºC for two hours. The various extracts of *A. nilotica* were diluted to obtain concentration ranging from 100 µg -500µg /ml. serial dilutions of plant extracts were made in 100 µl Mueller-Hilton broth. Subsequently, 10 µl of two hour old culture was added to each well. One well served as a medium control while other served as culture control. The plates were incubated for 24 hours at 37 ºC. The MIC was considered as the lowest concentration of the well that exhibited no bacterial growth as measured from turbidity of culture by optical density value.

Statistical analysis

The data were subjected to one way ANOVA to determine the significance of individual differences. Significant means were compared by the Duncan's multiple range tests. All the statistical analysis were carried out using SPSS software package (SPSS Version 16.0, Chicago, USA)

Results

Disc diffusion method

The antimycobacterial activity of various solvent extracts (Hexane, Chloroform, Butanol, Ethanol, Aqueous and active acetone fraction) of *A. nilotica* leaves was evaluated for zone of inhibition at different concentrations (50, 75, 100, 150 mg/mL) against *Mycobacterium smegmatis* by disc diffusion method. The result was tabulated in Table 5.2.1. The result showed increased zone of inhibition with increasing concentration. Graphical representation was presented in Fig 5.1.1. The zone of inhibition of standard dapsone at 50 mg/mL was 67 mm diameter. At 50mg/ml concentration, the acetone fraction showed maximum zone of inhibition at 44 mm diameter, followed by chloroform and butanol extract at 24 mm diameter. The next highest zone of inhibition was by hexane and ethanol extract at 22 mm diameter. The least zone was observed in aqueous extract at 21 mm diameter. At 75mg/ml concentration, the acetone fraction showed maximum zone of inhibition at 45 mm diameter. The next highest zone of inhibition was by chloroform extract at 33 mm diameter followed by aqueous extract at 24 mm diameter, Both hexane and ethanol extracts showed least zone at 23 mm diameter. At 100 mg/ml concentration, the acetone fraction showed maximum zone of inhibition at 48 mm diameter followed by chloroform extract at 41 mm diameter. Hexane extract showed the next highest zone of inhibition at 32 mm diameter. The next highest zone was formed by butanol extract at 25 mm diameter. Both ethanol and aqueous extract showed least zone of inhibition (24 mm). At 150 mg/ml the active fraction of acetone extract showed the maximum zone of inhibition at 52 mm diameter followed by chloroform extract with 50 mm diameter. The next highest inhibition zone was found in aqueous extract with 36 mm diameter. This was then followed by butanol, hexane and ethanol extract with diameters 34, 33 and 31 mm respectively. When the zone of

inhibition of all the extracts was compared statistically with standard Dapsone, the P value was significantly decreased (P<0.001).

Minimum inhibitory concentration test

Minimum Inhibitory Concentration was evaluated for different extracts of *A. nilotica* leaves (Hexane, chloroform, active acetone fraction, butanol, ethanol and aqueous) and compared with standard dapsone against *Mycobacterium smegmatis*. The results were tabulated in Table 5.2.2 The MIC of dapsone against *M. smegmatis* was 0.12 mg/ml. Active fraction of acetone extract showed lowest MIC at 0.15 mg/ml. Ethanol and hexane extract showed similar MIC at 0.21 mg/ml. It was then followed by butanol, aqueous and chloroform extracts at 0.28, 0.34 and 0.35 mg/ml (Fig 5.1.2) When MIC of all the extracts was compared statistically with standard Dapsone, the P value of all extracts were significantly increased (P<0.001).

Minimum bactericidal concentration test

Minimum Bactericidal Concentration was evaluated for different extracts of *A. nilotica* leaves (Hexane, chloroform, active acetone fraction, butanol, ethanol and aqueous) and compared with standard dapsone against Mycobacterium smegmatis. The results were tabulated in Table 5.2.3 The MBC of dapsone against *M. smegmatis* was 0.25 mg/ml. Acetone fraction showed lowest MBC at 0.28 mg/ml. Aqueous extract showed highest MBC at 0.47 mg/ml. The MBC value for other extracts such as hexane, chloroform, butanol and ethanol were 0.31, 0.42, 0.38 and 0.32 mg/ml respectively (Fig 5.1.3). When MBC of all the extracts was compared statistically with standard Dapsone, The P value of all extracts were highly significant (P<0.001) except acetone extract, where the P value was moderately significant (P<0.01).

Discussion

Leprosy is always a threat and one of the world's oldest disease and have tormented humans throughout history. The risk of this disease remains the same since its origination 5000 years ago because of the causative agent *M. leprae* easily gains resistance towards the antibiotic generated. Thus the need for the discovery of a novel drug has been increasing to eradicate the disease completely. The present work aimed at basic steps in identification of antileprosy drug from *A. nilotica*.

The antimycobacterial activity of extracts of *A. nilotica* leaves extract was performed against *M. smegmatis*, as *M. leprae* cannot be cultivated in laboratory conditions. The active acetone fraction *of A. nilotica* leaves showed best inhibiting activity compared to other extracts. It showed diameter of 52 mm zone of inhibition at 150 mg/ml concentration. Whereas the standard dapsone showed 67 mm zone of inhibition at 50 mg/ml. Acetone extract of *A.niotica* was not as effective as the standard drug but shows best inhibiting effect. The highest activity of standard dapsone may be due to the fact that it is in a highly purified form but the acetone extract of *A. nilotica* is in partially purified form. The dosage was calculated from the MIC and MBC that again showed acetone extract to be effective in least concentration of 0.15 – 0.28 mg/ml. When compared to other extracts, acetone extract of *A. nilotica* leaves has showed good inhibiting effect on *M. smegmatis*, It may also inhibit *M. leprae* effectively. However, further detailed *In vivo* studies are necessary to confirm the inhibitory effect of *A. nilotica* leaves acetone extract against *Mycobacterium leprae.*

Table 1: Antimicrobial activity of *A. nilotica* leaves extracts against *Mycobacterium smegmatis* by disc diffusion method

Solvent	Conc.(mg/ml)	Diameter of inhibition (mm)
Hexane	50	22 ± 0.2***
	75	23 ± 0.14***
	100	32 ± 0.2***
	150	33 ± 0***
Chloroform	50	24 ± 1.2***
	75	33± 0***
	100	41 ± 0.14***
	150	50 ± 0***
Acetone	50	44 ± 0.4***
	75	45 ± 0.1***
	100	48 ± 0.4***
	150	52 ± 0.3***
Butanol	50	24 ± 0.1***
	75	24 ± 0***
	100	25 ± 0.2***
	150	34 ± 0.14***
Ethanol	50	22 ± 0***
	75	23 ± 0.2***
	100	24 ± 0***
	150	31 ± 0***
Aqueous	50	21 ± 0.24***
	75	24 ± 0.24***
	100	24 ± 0***
	150	36 ± 0.28***
STD (Dapsone)	50	67 ± 0.24

Table 2: Minimum inhibitory concentration *A. nilotica* (leaves) extracts against *Mycobacterium smegmatis*

	Solvent						
	Hexane	**Chloroform**	**Acetone**	**Butanol**	**Ethanol**	**Aqueous**	**STD (Dapsone)**
MIC (mg/ml)	0.21±0.05***	0.35±0.03***	0.15±0.07**	0.28±0.06***	0.21±0.01***	0.34±0.07***	0.12±0

Table 3: Minimum bactericidal concentration of *A. nilotica* (leaves) extracts against *Mycobacterium smegmatis*

	Solvent						
	Hexane	**Chloroform**	**Acetone**	**Butanol**	**Ethanol**	**Aqueous**	**STD (Dapsone)**
MBC (mg/ml)	0.31±0.03***	0.42±0.01***	0.28±0.04**	0.38±0.02***	0.32±0.02***	0.47±0.03***	0.25±0

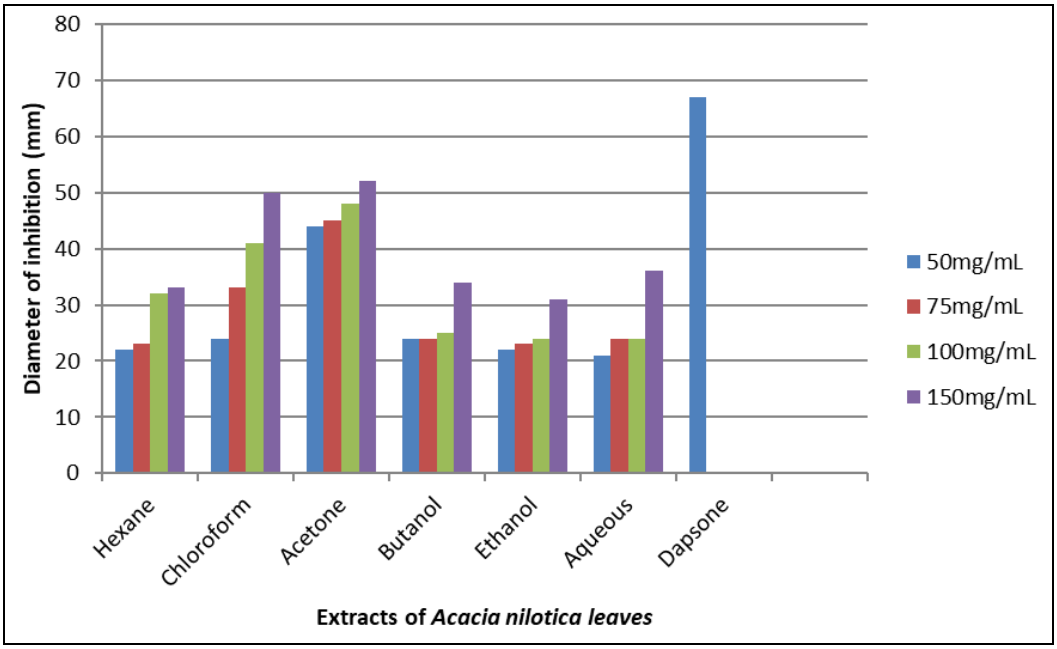

Fig 1: Antimycobacterial activity of active acetone fraction of *Acacia nilotica* leaves extract by Disc diffusion method against *M. smegmatis*

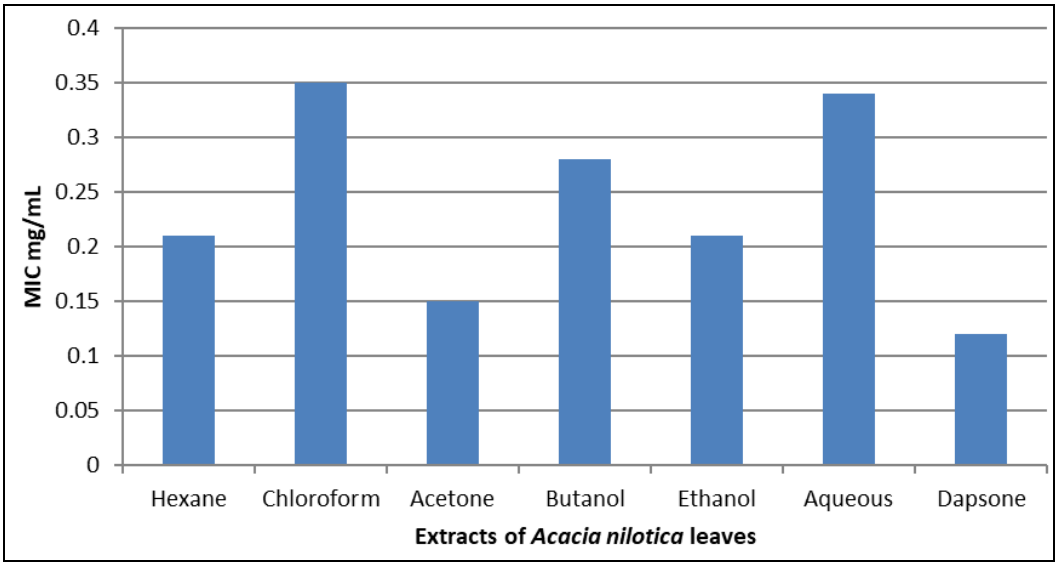

Fig 2: Minimum Inhibitory Concentration of *Acacia nilotica* leaves extracts against *M. smegmatis*

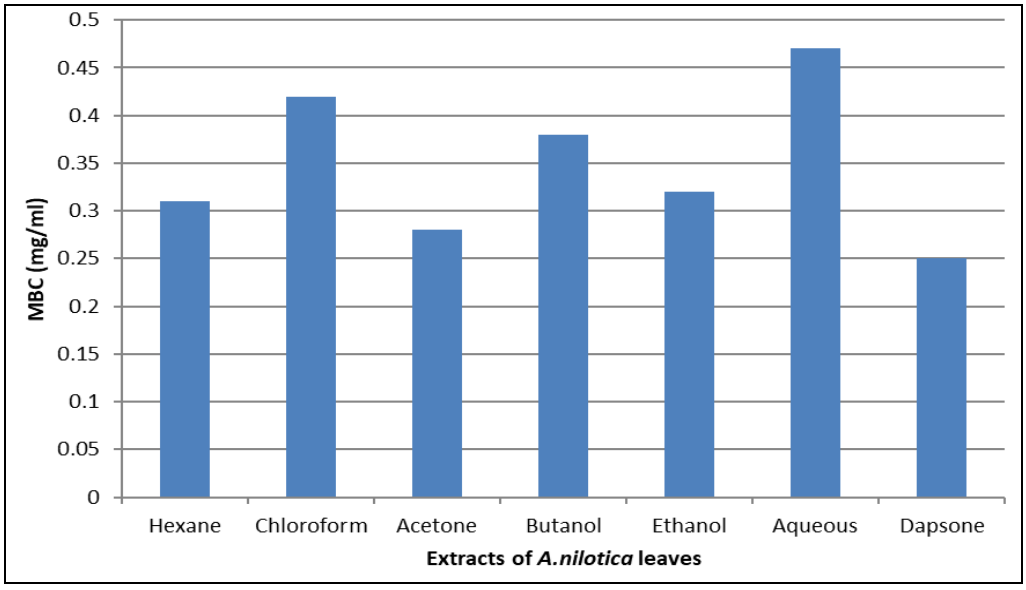

Fig 3: Minimum Bactericidal Concentration of *Acacia nilotica* leaves extracts against *M. smegmatis*

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