In-vitro Inhibitory Activity of Extracts of Some Medicinal Plants against *Mycobacterium smegmatis*

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Abstract: The current drugs used for the treatment of tuberculosis (TB) have become less effective due to the development of resistance by *Mycobacterium tuberculosis* to the drugs. As such there is a search for new drugs for treatment of TB. Medicinal plants used in traditional medicine for symptoms of TB in endemic countries represent potential sources of new compounds for drug development. This study was designed to investigate the antimycobacterial activities of extracts of some medicinal plants used in Abia State using the *Mycobacterium smegmatis* (ATCC 19420) model. Crude methanolic extracts and fractions of five medicinal plants (*Alchornea cordifolia*, *Asystacia gangetica*, *Chromolena odorata*, *Pterocarpus santalinoides* and *Garcinia kola*) were tested against a strain of *M. smegmatis* using the agar-well diffusion method to measure the diameter of zone of inhibition (DZI). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by the broth dilution method. Phytochemical screening of the plant materials was carried out using standard methods. The mean DZI of the methanol extracts ranged from 12.5mm to 18mm at a concentration of 100mg/ml. The extract of *G. kola* produced the highest mean DZI. The DZI produced by the ethyl acetate (EtAc) and dichloromethane: methanol (DCM:MeOH) fractions ranged from 7.5-14mm. The EtAc fraction of *A. cordifolia* produced a zone of inhibition of 14mm while all the three fractions (N-Hexane, EtAc and DCM:MeOH) of *C. odorata* produced inhibition zone of 12.0mm, 13.5mm and 11.5mm, respectively. The MIC values ranged from 25mg/ml to 50mg/ml. Preliminary qualitative phytochemical analysis showed that the plant extracts contained alkaloids, terpenoids, saponins, flavonoids and tannins. The thin layer chromatographic analyses suggested that the antimycobacterial activity was mainly due to the alkaloid constituents. *Garcinia kola* exhibited the highest activity, followed by *A. cordifolia* while the activity of *P. santalinoides* was the lowest. The results of this study demonstrate the *in-vitro* inhibitory activities of these medicinal plants against *M. smegmatis* and provide the basis for further studies to isolate and identify the compounds with antimycobacterial activity.

Key words: Medicinal plants, *Mycobacterium smegmatis*, antimycobacterial activity.

INTRODUCTION

Strains of pathogenic bacteria resistant to commonly available and affordable antimicrobial agents have emerged and now threaten the success of past decades of antimicrobial chemotherapy (Tanwar *et al.*, 2014). There is an urgent need to search for and develop new antibacterial agents that are effective against resistant bacteria. The need for new antibacterial agents is probably more urgent for tuberculosis (TB) caused by *Mycobacterium tuberculosis* complex than any other infectious bacterial disease (Chakraborty and Rhee, 2015). According to the World Health Organization Global TB Report 2017, there were 10.4 million cases of active TB diseases and 1.3 million deaths globally in 2016 with highest rates occurring in the Sub-Saharan Africa (WHO, 2017). The high global incidence of TB and the increasing problem of resistance to available anti-tuberculosis drugs make the search for new drugs for TB very urgent. Apart from the limited number of antimicrobial agents effective against *M. tuberculosis,* strains resistant to the most important first line and second line TB drugs, described as multidrug resistant (MDR) and extensively drug resistant (XDR), respectively have emerged and are spreading in different parts of the world (Zhang and Yew, 2015).

Plants have provided man with sources of foods and medicines from time immemorial. At the moment, medicines from plants remain an important component of the traditional system of healthcare, particularly in developing countries, where cost and availability limit access to orthodox medicines. In Nigeria, the use of varieties of herbal preparations to treat different kinds of ailments including microbial infections such as gonorrhea, respiratory tract infections, gastrointestinal infections and diarrhea is common (Anyanwu and Okoye, 2017). The enormous ethnomedical knowledge of the uses of medicinal plants in traditional system of medicine in developing countries is an important resource that needs to be explored for the purpose of discovering potential therapeutic agents. Increasingly, medicinal plants used in traditional medical practice are receiving greater attention in the search for novel compounds that can serve as scaffolds for new therapeutic compounds(Ogbole and Ajaiyeoba, 2010; Nguta *et al*., 2015). It has been shown that medicinal plants used in traditional medical practice have the potential to yield novel compounds with antimicrobial activities (Ncube *et al*., 2008).

Several studies have revealed that extracts of many medicinal plants used in traditional medicine in Africa had activity against different species of *Mycobacterium* including *M. bovis*, *M. vaccae*, *M. smegmatis* and the laboratory adapted *M. tuberculosis* strain H37Ra (Adeniyi *et al*., 2004; Mann *et al*., 2009; Mmushi *et al*., 2010; Nvau and Oladosu, 2011; Anochie *et al*., 2011; Gemechu *et al*., 2013). Due to lack of biosafety facilities to handle the highly infectious and very slow growing *M. tuberculosis,* the fast growing and nonpathogenic *M. smegmatis* has been found to be a safe and suitable surrogate for screening for antimycobacterial activities (Alloteybabington *et al*., 2014). In the present study, the antimycobacterial activities of extracts of five medicinal plants (*Alchornea cordifolia*, *Asystacia gangetica*, *Chromolena odorata*, *Pterocarpus santalinoides* and *Garcinia kola*) used for treatment of various ailments in Abia State were investigated.

MATERIALS AND METHODS

The fresh leaves of the medicinal plants (*A. cordifolia*, *A. gangetica*, *C. odorata*, *P. santalinoides* and *G. kola*) were collected from three different Local Government Areas (Isuikwuato, Bende and Ikwuano) in Abia State. The medicinal plants were authenticated by a Plant Taxonomist in the Department of Forestry and Environmental Management, Michael Okpara University of Agriculture Umudike, Abia State. The plant materials were air dried for two weeks on cardboards on the floor of a well-ventilated shed used for drying plant materials. The dried parts were pulverized to fine powder using a mechanical grinder. The powdered leaf materials were sieved, weighed and stored in airtight containers at room temperature until they were extracted.

Extraction of plant materials.

Five hundred grams (500g) of each of the powdered plant materials was soaked in 1,500ml of methanol for 24h at room temperature. The extracts were filtered using non-adsorbent muslin cloth into a clean beaker. The filtrate was dried by evaporating off the solvent at 50^{o}C in a hot air oven over a period of one to two days.

Phytochemical Screening

Qualitative phytochemical screening of the plant materials for Saponins, Flavonoids, Alkaloids, Tannins and Terpenoids was done as described by Harbone (1973).

Thin Layer Chromatography

The Thin Layer Chromatography (TLC) analysis of the extracts was carried out using Whatman TLC plates precoated with KS Silica gel (Whatman Limited, Maidstone, England). An aliquot of 5µl of each of the extract solutions in methanol was spotted at position 1cm from the bottom of TLC plate using a capillary spotter.

The plates were developed in a developing chamber using suitable solvent combinations. The chromatograms were dried and detection was made using ultraviolet light at wavelengths of 254nm and 365nm.

Fractionation of the methanolic extracts

The methanolic extracts were fractionated by column chromatography on silica gel (60-200 mesh) packed into a glass column (45cm ×2cm) to about half the length to obtain the various fractions (ethyl acetate (EtAc), dichloromethane: methanol (DCM: MeOH) and hexane). A slurry of methanol extract and silica gel was prepared by mixing 20g of Silica gel with 8g of extract dissolved in 2ml of methanol. The slurry was spread on the top of the packed silica gel in the column. A small wad of cotton wool was placed on the surface of the column to prevent any disturbance from solvent delivery to the packed column. The column was successively eluted with N-Hexane (100%), Dichloromethane: methanol (80:20%) and Ethyl-acetate (100%). Fifteen fractions of 50ml each were collected.

Screening of extracts and fractions for antimycobacterial activity

The antimycobacterial activities of the extracts were assessed by the agar well diffusion assay as previously described(Allotey-babington *et al.*, 2014) with slight modifications. Briefly, a stock solution of 100mg/ml of each of the plant extracts was made in dimethyl sulfoxide (DMSO). Further dilutions were made to obtain concentrations of 50mg/ml and 25mg/ml. The test organism, *M. smegmatis* ATCC 19420, was reactivated by streaking out on a freshly prepared nutrient agar plate and stored on agar slant according to the manufacturer's instructions. An aliquot of 100µl of suspension of *M. smegmatis* standardized to 0.5 MacFarland standard (approximately 10^8 CFU/ml) was aseptically inoculated by spread plate technique unto nutrient agar surface in a sterile Petri dish. Wells were created on the agar surface using a sterile cork-borer of 6 mm diameter. An aliquot of 50µl of each of the plant extracts was loaded into each well. The plates were incubated at 37°C for 48 h. The diameter of the zone of inhibition was measured in mm using a transparent plastic ruler. The EtAc, DCM: MeOH and hexane fractions were similarly tested. Rifampicin was used as a positive drug control while DMSO was used for solvent control.

The minimum inhibitory concentration (MIC) was determined by macrobroth dilution technique. Serial dilutions of the plant extracts were made in test tubes containing sterile Mueller Hinton Broth, to obtain concentrations of 100, 50, 25, 12.5, 6.25, 3.12 and 1.56mg/ml). The test tubes were inoculated with 50µl of suspension of the test bacterium standardized to McFarland Standard tube No.0.5. The inoculated tubes were incubated aerobically at 37°C for 18-24 h. After incubation, the tubes were examined for turbidity. The tube with the lowest concentration of extracts which showed no turbidity was recorded as the MIC value for the tested extract. The minimum bactericidal concentration (MBC) was determined by streaking the contents of the two last tubes with no turbidity, separately, on freshly prepared nutrient agar plates. The MBC is the concentration in the tube from which no growth was observed after 18-24 h of incubation.

RESULTS

The antimycobacterial activities of the methanol extracts of each of the five medicinal plants were assessed using the diameter of zone of inhibition produced in agar well diffusion assay and minimum inhibitory concentration (MIC) as well as the minimum bactericidal concentration (MBC) in broth dilution assay. The results are presented in Table 1. The extracts of the five medicinal plants produced mean diameter of inhibition zones ranging from 12.5 mm to 18 mm at concentration of 100mg/ml. The MIC values ranged from 25mg/ml to 50mg/ml and MBC values from 50mg/ml to 100mg/ml. The methanol extracts of *G. kola*, *A. cordifolia* and *C. odorata* pro-

duced the highest zones of inhibition of 18mm, 17.5mm and 16.5mm, respectively.

The results of the inhibition zones produced by N-Hexane, Ethyl acetate and Dichloromethane-Methanol (DCM-MeOH) fractions are presented in Table 2. The ethyl acetate and DCM: MeOH fractions of the extracts produced varying levels of inhibition ranging from 9mm to 14mm for Ethyl acetate and 7.5mm to 13mm for DCM: MeOH at the highest concentration of 100mg/ml. The N-Hexane fractions of all the plants did not show any activity except that of *C. odorata* with a zone of inhibition of 12mm at 100mg/ml. The results of the TLC analysis and semi- quantitative phytochemical screening of the medicinal plants is presented in Table 3. The major phytochemical constituents detected at various levels include alkaloids, flavonoids, saponins, terpenoids and tannins. Semi-quantitative grading of the strength of detection showed different levels of the presence of these phytochemicals in the fractions. The TLC analyses produced different numbers of spots ranging from one to four with different RF values. The diameter of zone of inhibitions of the various fractions are indicated in Table 3.

Table 1: Antimycobacterial activity of methanol extracts of medicinal plants against *M. smegmatis*

Plant Extracts	Concentrations (mg/ml)	Mean diameter zone of inhibition (mm)*	Minimum Inhibitory Concentration (MIC) (mg/ml)	Minimum Bactericidal Concentration (MBC) (mg/ml)
Alchornea cordifolia	100	17.5±0.70	25	50
	50	11.0±1.41		
	25	0.0±0.00		
Asystacia gangetica	100	15.5 ±0.70	50	100
	50	12.5±0.70		
	25	9.5±0.00		
Chromolena odorata	100	16.5±0.70	25	50
	50	12.5±2.12		
	25	0.0±0.00		
Garcinia kola	100	18.0±1.41	50	100
	50	10.5±0.70		
	25	0.0±0.00		
Pterocarpus santalinoides	100	12.5±0.70	50	100
	50	10.0±0.00		
	25	0.0±0.00		
Rifampicin		32.5±2.12		

*Values in the table are mean ± standard deviation of two replications of diameter of zones of inhibition (in mm).

Table 2: Diameter of zone of inhibition(mm) produced by various fractions of methanol extracts against *Mycobacterium smegmatis*

		Concentration(mg/ml)		
Plants	Fractions	100	50	25
Alchornea cordifolia	N-Hexane	0.0±0.00*	0.0±0.00	0.0±0.00
	Ethyl acetate	14.0±0.00	11.0±1.41	0.0±0.00
	DCM: MeOH	12.5±0.70	0.0±0.00	0.0±0.00
Asystacia gangetica	N-Hexane	0.0±0.00	0.0±0.00	0.0±0.00
	Ethyl acetate	12.0±1.41	8.0±0.00	0.0±0.00
	DCM: MeOH	13.0±0.00	10.0±0.00	0.0±0.00
Chromolena odorata	N-Hexane	12.0±1.41	0.0±0.00	0.0±0.00
	Ethyl acetate	13.5±0.70	12.0±0.00	0.0±0.00
	DCM: MeOH	11.5±0.70	10.5±0.70	0.0±0.00
Garcinia cola	N-Hexane	0.0±0.00	0.0±0.00	0.0±0.00
	Ethyl acetate	9.0±1.41	0.0±0.00	0.0±0.00
	DCM: MeOH	12.0±0.00	9. 0±1.41	0.0±0.00
Pterocarpus santalinoides	N-Hexane	0.0±0.00	0.0±0.00	0.0±0.00
	Ethyl acetate	10±0.00	0.0±0.00	0.0±0.00
	DCM: MeOH	7.5±0.70	0.0±0.00	0.0±0.00
Rifampicin		32.5±2.12		

DCM: MeOH = Dichloromethane: Methanol

*Values in the table are mean ± standard deviation of two replications of diameter of zones of inhibition (in mm).

Table 3: TLC fingerprints, phytochemical detection and antibacterial activity of fractions of extracts against *M. smegmatis*

Plant (Fraction)	№. of Spots	RF values	Detection of phytochemicals in extracts					Zone of inhibition (mm)
			Alkaloid	Terpenoid	Saponin	Flavonoid	Tannin	
Alchornea cordifolia (Ethylacetate)**	3	0.51 0.75 0.82	++	++	++	+	+	14.00±00
Asystacia gangetica (Ethyl Acetate -MeOH)	2	0.46 0.50	++	-	++	+	+	12±1.41
Chromolena odorata (Ethylacetate)	2	0.36 0.49	++	+	+	+	+	13.5±0.70
Garcinia kola (DCM-MeOH)	4	0.20 0.31 0.38 0.45	++	+	++	++	++	12.00±00
Pterocarpus santalinoides (Ethyl Acetate-MOH)	1	0.55	-	++	+	++	++	10.0±00

** Solvent or solvent combination used for mobile phase
++ Strongly detected
+ Weakly detected
- Not detected

DISCUSSION

Tuberculosis (TB) is an ancient disease that has plagued the human race for centuries. It is well known in different cultures around the world. As such there is an enormous ethnomedicinal knowledge of the use of medicinal plants in the treatment of TB around the world (Adegboye *et al*., 2002). In recent times, many investigators have turned their attention to medicinal plants in search of novel compounds for development of new anti-tuberculosis drugs. The five medicinal plants evaluated in this study exhibited inhibitory activity against *M. smegmatis*. The crude methanol extracts of *G. kola*, *A. cordifolia*, *A. gangetica* and *C. odorata* were the most active, producing high inhibitory activities ranging from 15.5mm to 18mm at concentration of 100mg/ml and 10mm to 12.5mm at 50mg/ml. Several researchers have reported interesting antimicrobial properties of these medicinal plants in different investigations (Adegboye *et al*., 2002; Ukaoma *et al*., 2013; Chic and Amom, 2014; Nwokorie *et al*., 2015).

However, none of these investigators reported on the antimycobacterial activities of these plants although ethnobotanical surveys reported the use of some of them in the treatment of tuberculosis or coughs **(**Ogbole and Ajaiyeoba,2010; Nguta *et al*., 2015**).** The methanol extract of the leaves of *G. kola* produced the highest zone of inhibition of 18mm. It is interesting that the leaf extract of *G. kola* had high antimycobacterial activity. Most investigations have focused on the seed which is what is mostly used in ethnomedicine. However, the leaves are more readily available, cheaper and more sustainable to harvest and work with. Our results agree with those reported by Gupta *et al*. (2016) on the activity of some medicinal plants from Ghana against *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*.

Although the antimycobacterial activities of many medicinal plants have been investigated, the use of different models and methods lead to different results which are difficult to compare. The ideal test organism for antimycobacterial activity testing is *M. tuberculosis*; the real causative agent of TB. However, the handling of *M. tuberculosis* requires level III biosafety infrastructure and high level of training. This is not usually widely available, especially in less industrialized countries. Further, *M. tuberculosis* grows very slowly and formation of visible colonies could take from two to eight weeks. These factors make the direct use of *M. tuberculosis* in antimycobacterial screening difficult and requires methods that cannot be performed in a laboratory with basic infrastructure. The use of the non-pathogenic rapidly growing *M. smegmatis* permits the use of low-cost rapid methods like well diffusion method which produces zone of inhibition that can be easily quantified. This model has been validated by some authors as a suitable surrogate for antimycobacterial screening (Altaf *et al*., 2010). It has also been noted that at least 12 of the 19 known virulence genes of *M. tuberculosis* share close homology with genes of *M. smegmatis* (Barry *et al*., 2009).

The antimycobacterial activities of three column chromatographic fractions (N-Hexane, Ethyl acetate and Dichloromethane: methanol (DCM: MeOH) of the methanol extract of each of the plants was further tested. The Ethyl acetate and DCM: MeOH fractions of all the plants were active but produced zone of inhibition lower than those of the crude extracts. This suggest that the activity in the crude extracts may be due to the presence of several constituents acting synergistically or there was a loss in the concentration of the active constituents in the process of fractionation. The detection of several phytochemicals in the extracts and the presence of multiple spots in the thin layer chromatographic (TLC) analyses appear to support this notion. Other workers have observed that antimicrobial activity of medicinal plants is often due to the presence of several constituents (Cowan,1999, Mbah *et al*., 2012). The N-Hexane fraction did not produce any zone of inhibition except that of *C. odorata*. The lack of antimycobacterial activity observed for the N -Hexane fractions suggests that the active metabolites of these plant species may not be lipophilic or could not diffuse adequately through the agar gel in the test system.

CONCLUSION

This study has shown that the extracts of the medicinal plants (*A. cordifolia*, *A. gangetica*, *C. odorata*, *P. santalinoides* and *G. kola*) exhibited appreciable inhibitory activities against *M. smegmatis* which was used as a surrogate for *M. tuberculosis*. As such, the medicinal plants deserve further studies in a more advanced laboratory where the activities can be directly evaluated against pathogenic *M. tuberculosis* and to also isolate and identify the active compounds.

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