ANTIMYCOBACTERIAL ACTIVITIES OF TERPENES/STEROLS FROM HEXANE AND TANNINS AND FLAVONOIDS FROM METHANOL EXTRACTS OF *PIPER GUINIENSE* LINN. (*PIPERACEAE*) SEEDS

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Abstract

Piper guiniense seeds used to treat tuberculosis and/or its symptoms were screened for *in vitro* activity against *M. smegmatis* ATCC 607, *M. tuberculosis, M. bovis* and *M. avium-complex*. A preliminary screening of the crude extracts obtained by sequential solvent extraction was done by the proportion method. The hexane extract inhibited *M. smegmatis* ATCC 607 and *M. tuberculosis* at a concentration of 1.0 mg/ml. The methanol extract showed inhibitory activity against *M. smegmatis* ATCC 607, *M. tuberculosis* and *M. bovis* at concentrations of 0.5 mg/ml, 1.0 mg/ml and 5.0 mg/ml respectively. The two crude extracts were subjected to bioassay-guided fractionation, using solvent/solvent separation, column chromatography and thin layer chromatography (TLC) for the methanol extract, while fractional distillation was used for the hexane extract. The isolated active fractions were screened for major chemical groups using TLC on pre-coated silica (silica gel G_{F254}) TLC plates (Merck, Germany. Terpenes/sterols were detected in the fraction from the hexane crude and, tannins and flavonoids in the fraction from the methanol crude extract.

Keywords: Antimycobacterial activity, terpenes/sterols, tannins, flavonoids, *Piper guiniense* seeds.

1. Introduction

The indiscriminate use of antibiotics has led to the emergence of drug resistant bacteria (Piddock and Wise, 1989; Singh *et al.*, 1992; Mulligen *et al.*, 1993; Davies, 1994; Robin *et al.*, 1998). This complicates the treatment of infectious diseases especially those associated with Acquired Immune Deficiency Syndrome (AIDS) (Rinaldi, 1991; Diamond, 1993). Tuberculosis (TB), a very important opportunistic infection in AIDS patients remains a serious public health problem especially in the developing nations. The control and prevention of the TB epidemic will depend largely on adequate treatment, and possibly on effective chemo prophylaxis (WHO/IUATLD Working Group, 1989). The present treatment regimes are based on multi-drug therapy usually with three or four antituberculosis drugs. An ideal TB treatment regime must contain multiple drugs to which the organisms are susceptible. Multi-drug resistant Tubercle bacilli have emerged for various anti-TB drugs, such as isoniazid, ethambutol, rifampicin and streptomycin (Girling, 1989; Grange and Davey, 1990). Resistance to such drugs has been reported by the WHO (1997) to be on the increase over the years. The same report indicated that globally two percent of all cases of TB are multi-drug resistant.

Drug resistance by *Mycobacterium tuberculosis* and other atypical Mycobacteria pose serious problems to local and global TB control programmes. This is because drug-resistant TB is very difficult to treat and requires more and different medications for a longer period of treatment; it is more toxic, thereby raising serious problems of compliance; more expensive and; not as successful (American Thoracic Society, 1992). In drug-resistant TB, surgery is sometimes needed to remove areas of destroyed lungs that are heavily infected by Mycobacteria (National Jewish Medical and Research Center, 1994). This adds heavily to the cost of treatment. The present scenario makes the search for novel antibacterial agents active against *Mycobacterium tuberculosis* and other atypical mycobacteria urgent. Plants being a biologically and chemically diverse resource that constitutes an effective source of both traditional and modern medicine may provide and alternative or an important lead for the development of anti-tuberculosis agent active against multi-drug resistant tubercle bacilli.

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The genus *Piper* (Family: *Piperaceae*) contains several species some of which are reported to possess important biological activity and application in traditional medicine. Abbas (2002; personal communication) revealed that *Piper guiniense* mixed with honey is used as a remedy for TB and TB like symptoms such as cough and blood in the sputum. Lentz *et al.*, (1998) reported the use of *Piper aduncum* in the traditional pharmacopia of the Pech and Mestizo people of Honduras. The plant also revealed signs of antifungal activity (Lentz *et al.*, 1998). The fruits of some selected *Piper species* (*P. chaba* Hunt, *P. longum* Linn and *P. nigrum* Linn) exhibited marked antioxidant activity (Tewtrakul, 1998). Scott *et al.*, 2002 isolated a black pepper, *Piper nigrum* constituent, piperine that exhibited insecicidal activity against three species namely *Periplaneta americana, Musca domestica* and *Leptinotarsa decemlineata.* The plant species also exhibited activity against *Pseudomonas aeruginosa, Bacillus subtilis* and *Staphylococcus aureus* (Valsaraj *et al.*, 1997). Despite these biological activities and the use of *Piper* species in traditional medicine especially for the treatment of tuberculosis and/or its symptoms, there hasn't any study to determine the antimycobacterial activities of the plant. This paper therefore reports on the antimycobacterial activity and phytochemical screening of extracts of seeds of *Piper guiniense.*

2. Materials and Methods

(a) Collection of plant samples

The seeds of *Piper guiniense* were bought from Muda Lawal market, Bauchi, Nigeria. The plant was identified at the herbarium of the Biological Sciences Programme, Abubakar Tafawa Balewa University (A.T.B.U.) Bauchi, Nigeria. The identity of the plant was finally confirmed at the herbarium of the National Institute of Pharmaceutical Research and Development (NIPRD), Idu-Abuja, Nigeria where herbarium specimen was deposited (NIPRD 5386). The seeds were grounded into fine powder using pestle and mortar.

(b) Test organisms

Four species of *Mycobacterium* were used for the study. These are *Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium avium-complex* and *Mycobacterium smegmatis* ATCC 607. The first three species were isolated from sputum samples obtained from patients at Tuberculosis and Leprosy Hospital Bayara in Bauchi. Isolation was done according to standard procedures on Glycerol-Egg and Lowenstein-Jensen (L-J) media (Baker *et al.*, 1998). *Mycobacterium smegmatis* ATCC 607 was obtained from Microbiology Department of the National Institute for Pharmaceutical Research and Development, Idu-Abuja.

(c) Extraction procedure

(i) Sequential solvent plant extraction

The plant material was subjected to sequential solvent extraction. This was done according to the methodology described by Pistelli *et al.* (2000) and Newton *et al.* (2002). One kg of the plant sample was soaked in 2.5 L of hexane and allowed to stand for 72 hours with vigorous shaking after every 24 hours. At the end of extraction the extract was filtered through Whatman No. 1 filter paper (England). Subsequently the residue was extracted with chloroform, acetone, methanol and water in that order. The extract was concentrated to dryness at <40° C using a rotary evaporator (Janke & Kunkee, Labortechnik). The dried extracts were assessed for *in vitro* antimycobacterial activity.

(ii) Determination of Antimycobacterial Activity

Each plant extract was tested for antimycobacterial activity using the agar plate (proportion) method of Canetti *et al.* (1969), Vareldzis *et al.* (1993) and, Lall and Meyer (1999). The proportion method determines the proportion of the bacterial population that is resistant to the plant extract tested. When 1% or more of bacteria tested are resistant to the drug (plant extract), the population is considered resistant.

(iii) Preparation of inoculum for the agar plate (proportion) method

Standard inoculum was prepared in physiological saline containing 0.5% Tween 80 to obtain a concentration of 1mg/ml (wet mass) according to the method described by Lall and Meyer (1999). Growth of the bacilli to be tested was picked from the culture slope using a sterile applicator stick. This was transferred into a sterile screw-capped bottle containing 6-8 glass beads and 4 ml of physiological saline containing 0.5% Tween 80. The mixture was homogenized by shaking for 5 minutes. Large particles were allowed to settle and more broth was added and turbidity adjusted to McFarland no. 1 standard. This suspension was diluted to $1x10^{-2}$ mg/ml and $1x10^{-4}$ mg/ml. To each bottle containing plant extract (10 mg/ml), 0.2 ml of the $1x10^{-2}$ mg/ml inoculum was inoculated. For the control (medium without plant extract), 0.2 ml of each of the $1x10^{-2}$ and $1x10^{-4}$ mg/ml were inoculated. All tubes were incubated at 37° C for 8 weeks. The experiments were done in duplicates.

(d) Interpretation of results

The number of colonies that were growing on the medium with the plant extracts, N^{-2} for the dilution $1x10^{-2}$ mg/ml, was compared with the growth in the control series, (no plant extracts), NO^{-2} for $1x10^{-2}$ mg/ml and NO^{-4} for $1x10^{-4}$ mg/ml. The following criteria were used for the interpretation of results (Lall and Meyer, 1999). If:

(i) $N^{-2} \geq NO^{-2}$ = Resistant.

(ii) $NO^{-4} \leq N^{-2} \leq NO^{-2}$ = Partially Susceptible.
(iii) $N^{-2} \leq NO^{-4}$ = Sensitive (< 1% growth).

(e) Isolation of active fraction from *Piper guiniense* seeds methanol and hexane crude extracts

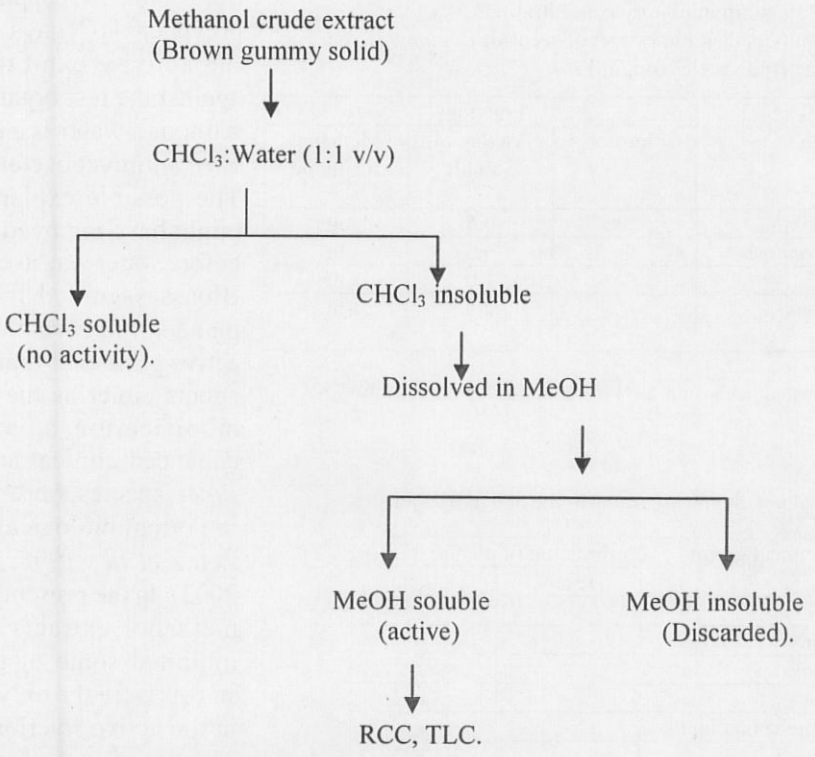
The methanol fraction was subjected to solvent/ solvent separation (Figure 1). The active fraction was further subjected to column chromatography over silica gel and eluted with ethyl acetate and ethyl acetate:methanol (2:1). Fractions were monitored by TLC (Hexane:Ethylacetate:Methanol) [12:3:2], detecting with Dragendorff's spray reagent.
The hexane extract (a brownish oil) also exhibited *in vitro* antimycobacterial activity. The oil was subjected to fractional distillation and the fractions were further screened for *in vitro* antimycobacterial activity. The active fraction was tested for major chemical groups using TLC.

(f) Identification of Major Chemical Groups in the Isolated Active Fractions

The identification of major chemical groups in the active fractions was carried out by TLC on pre-coated silica (silica gel G_{F254}) TLC plates (Merck, Germany). For terpenes and sterols, hexane:ethyl acetate: 1:1 was used as a mobile phase and Liebermann-Burchard as reagent, a range of colours were produced after heating sprayed plates for 10 min at 100°C (Tona *et al.*, 1998). Alkaloids were detected by acid/base extraction procedure for alkaloids and analyzed by TLC in chloroform/methanol/ammonia solution 25% 8:2:0.5 as solvent system, spots were detected after spraying with Dragendorff's reagent. For the detection of flavonoids, TLC was developed in n-butanol/acetic acid/water 4:1:5. Spots were visualized with 1% aluminium chloride solution in methanol under ultra violet light (Harbone, 1974). Aqueous extracts were used for the detection of tannins with 1% gelatin solution and saponins by froth test (Tanira *et al.*, 1994).
Flavanols and flavanol-3-glycosides were detected with 0.5g of metallic zinc and 10 drops of concentrated sulphuric acid (H_2SO_4); reducing compounds were detected by Benedict's test and, Cardiac glycosides detected by dissolving 0.5g of sample in 2 ml of chloroform and a few drops of concentrated H_2SO_4 added (Harbone, 1974; Trease and Evans, 1978).

3. Results

The minimum inhibitory concentrations of the crude extracts obtained by sequential solvent extraction of seeds of *P. guiniense* are shown in Table 1. Hexane extract exhibited activity against *M. smegmatis* ATCC 607 and *M. tuberculosis* with MIC of 1.0 mg/ml, while the methanol extract inhibited the growth of *M. smegmatis* ATCC 607, *M. tuberculosis* and *M. bovis* with MICs of 1.0 mg/ml, 0.5 mg/ml and 5.0 mg/ml respectively. The chloroform, acetone and



$CHCl_3$ = chloroform; MeOH = methanol; RCC = repeated column chromatography; TLC = thin lay r chro tography; MeOH insoluble not tested because material contained hard particles.

Figure 1: Fractionation of methanol fraction of *Piper guiniense* seeds (Agarwal *et al.*, 2000).

water extracts did not show activity against any of the test organisms in this screening.

The results of the screening of the isolated active fraction from the methanol crude are shown in Table 2. Only the methanol soluble fraction exhibited activity inhibiting the four test organisms with MICs of 0.5 mg/ml, 0.5 mg/ml, 1.0 mg/ml and 2.5 mg/ml against *M. smegmatis* ATCC 607, *M. tuberculosis*, *M. bovis* and *M. avium-complex* respectively. The MIC of the fraction obtained from the hexane extract after distillation remained 1.0 mg/ml.

The major chemical groups in the isolated active fractions are shown in Table 3. Terpenes/sterols were detected in the fraction from hexane crude while tannins and flavonoids were detected in the fraction from methanol extract.

Table 1: Minimum inhibitory concentrations of crude extracts obtained by sequential extractionof seeds of *P. guiniense* on 4 Mycobacterial species (mg/ml).

Plant extract	Test Organisms			
	Msmg	Mtb	Mbv	Mac
Hexane	1.0	1.0	-	-
Chloroform	-	-	-	-
Acetone	-	-	-	-
Methanol	1.0	0.5	5.0	-
Water	-	-	-	-

Msmg = M. smegmatis; Mtb = M. tuberculosis, Mbv = M. bovis; Mac = M. avium-complex; - = Absence of in vitro activity.

Table 2: Minimum inhibitory concentrations of active fraction from methanolic extract of seeds of *P. guiniense* on 4 Mycobacterial species (mg/ml).

Test Organisms	Fractions		
	Chloroform	Methanol Soluble	Methanol Insoluble
M. smegmatis	-	0.5	nt
M. tuberculosis	-	0.5	nt
M. bovis	-	1.0	nt
M. avium-complex	-	2.5	nt

nt = not tested because material was hard; - = no activity

Table 3: Major chemical groups in the active fractions

Chemical group	Fraction of plan extract	
	Hexane	Methanol
Alkaloids	-	-
Saponins	-	-
C-glycosides	-	-
Tannins	-	+
Reducing compounds	-	-
Flavonoids	-	+
Flavanols	-	-
Terpenes/Sterols	+	-

+ = present; - = absent

4. Discussion

The methanol and hexane extracts of *P. guiniense* seeds obtained by sequential extraction were found to possess *in vitro* antimycobacterial activity in the present study. Although previous studies have not reported the antimycobacterial activity of this plant species, reports indicated that the plants possesse biological activities. For instance *Piper* species have been reported by Valsaraj *et al.*, (1997) to possess *in vitro* activity against both Gram positive and negative bacteria.

The MIC value of the hexane fraction on the test organisms was found to be 1.0 mg/ml. A previous study by Newton *et al.*, (2002) indicated that hexane extract of *Psoralea corytifolia* from sequential solvent extraction was found to be the most active against the *Mycobacterium* species tested (MIC = 31.25 µg/ml). Although hexane is a non-polar solvent, it can extract biologically active components such as terpenes (Cowan, 1999).

Among the extracts obtained by sequential solvent extraction, the methanol fraction was found to be the most active against the test organisms (Table 1). The MIC values ranged between 0.5 mg/ml and 5.0 mg/ml. The activity of the methanol fraction may be due to the fact that methanol being a polar solvent extracts a wide range of bioactive plants compounds such as terpenoids and tannins (Taylor *et al.*, 1996), quassinoids (Kitagawa *et al.*, 1996), lactones (Rao *et al.*, 1993), flavones (Taniguchi and Kubo, 1993; Sato *et al.*, 1996), phenones (Peres *et al.*, 1997) and polyphenols (Vijaya *et al.*, 1995). This might explain the activity exhibited by the methanol fraction against the test organisms in this study.

Although water is a polar solvent, it exhibited no *in vitro* antimycobacterial activity in the present study. The possible explanation might be that methanol might have removed most of the active components before water was used as reported by Cowan (1999). Bioassay-guided fractionation used in screening plants for *in vitro* bioactivity lead to the isolation of active principles that may be developed into clinical agents either as the natural product or a synthetic modification or a synthesized analogue with enhanced clinical action and reduced side effects. *Piper* species were previously reported to possess important biological activity (Valsaraj *et al.*, 1997; Lentz *et al.*, 1998; Tewtrakul, 1998; Scott *et al.*, 2002). In the present study, fraction from hexane and methanol extracts of the seeds of *P. guiniense* inhibited some of the test organisms. Terpenes/ sterols were the only groups of compounds detected in the active fraction isolated from hexane extract. The activity of this fraction could be due to the activity of the terpenes/sterols. Terpenes were reported by Cowan (1999) to possess activity against *M. tuberculosis*. Tannins and flavonoids contained in the fraction isolated from methanol extract of seeds

of *P. guiniense* might be responsible for its activity against the test organisms.

Tannins were also eported to have a wide range of anti-infective activity, toxic to filamentous fungi, yeasts and bacteria (Scalbert, 1991; Haslam, 1996; Stern *et al.*, 1996). A study by Ibrahim *et al.* (1997) on the antimicrobial activity of phytocompounds indicates that tannins showed greatest effect followed by flavonoids. The results of this study show that the antimycobacterial activity exhibited by the seeds extract may be attributable to the presence of these phytocompounds.

Terpenes were reported to possess activity against bacteria and fungi (Vishwakarma, 1990; Ahmed *et al.*, 1993; Harrigan *et al.*, 1993; Kubo *et al.*, 1993; Fujioka and Kashiwada, 1994; Hasegawa *et al.*, 1994; Ayafor *et al.*, 1994; Pengsuparp, 1994; Goshal *et al.*, 1996; Barre *et al.*, 1997; Amaral *et al.*, 1998). The mode of action of terpenes involves membrane disruption by lipophilic compounds (Mendoza *et al.*, 1997). This might explain the *in vitro* inhibitory activity exhibited by the hexane fraction on the test organisms considering the fact that the test organisms have high lipid content in their cell walls. Flavonoids have also been reported to have *in vitro* antimicrobial activity against a wide range of microorganisms because lipophilic flavonoids disrupt microbial membranes (Tsuchiya *et al.*, 1994).

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