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Evaluation of *in vitro* antimycobacterial activity of Nigerian plants used for treatment of respiratory diseases

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The global threat of tuberculosis (TB) demands for search for alternative antimycobacterial drugs. Some Nigerian medicinal plants used in the treatment of TB and other respiratory diseases were evaluated for activity against a clinical isolate of *Mycobacterium tuberculosis* and a strain of *Mycobacterium bovis* (BCG). The crude methanolic extracts of eight plant species were screened for activity against a clinical isolate of *M. tuberculosis* using broth microdilution method. Four out of the eight plant extracts exhibited inhibitory activities against *M. tuberculosis* at 78 and 1250 µg/mL. The crude extracts of *Entada africana*, *Hymenocardia acida*, *Sterculia setigera* and *Stereospermum kunthianum* did not inhibit significantly even at high concentration of 1250 µg/mL. The hexane fractions obtained after fractionation were the most active fractions for all the plants tested against BCG, having *Anogeissus leiocarpus* and *Terminalia avicennioides* exhibiting the highest activity at 312 and 200 µg/mL, respectively. Fractions Ta5 and A14 obtained on further purification exhibited most significant activity (MIC of 4.7 and 7.8 µg/mL, respectively). From the results of phytochemical analysis, terpenes and triterpenoid saponins are the most prominent compounds in these fractions and several reports earlier indicated that these metabolites are potential antimycobacterial agents. This class of metabolites presents interesting area for further investigation with special attention on the Combretaceae family from Nigeria flora.

Key words: Antimycobacterial activity, antimycobacterial agents, *Mycobacterium tuberculosis*, Nigerian medicinal plants, tuberculosis.

INTRODUCTION

Respiratory diseases are among the major human killers in the recent years. This is attributed to increases in respiratory tract infections and HIV/AIDS (Iwu et al., 1999).

This negative health trend rekindled interest in respiratory infectious disease research with reference to the development of drugs.

Plants have long been a valuable source of novel drug compounds (medicines). Phytomedicines, which are plant-derived, have shown great promise in the treatment of intractable infectious diseases including tuberculosis (TB) (Cowan, 1999; Mitscher and Baker, 1998). The populations of developing countries worldwide continue to

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to rely heavily on the use of traditional medicines as their primary source of health care. Ethnobotanical studies carried out throughout Africa confirm that indigenous plants are the main constituents of traditional African medicines (Adjanohoun et al., 1991; Hedberg et al., 1983a, b; Kokwaro, 1976; Mann, 1998; Oliver-Bever, 1986). With 70 - 80% of Africa's population relying on traditional medicines, the importance of the role of medicinal plants in the health care delivery is very enormous particularly for the respiratory diseases.

Farnsworth (1990) claims that 119 characterized drugs are still obtained commercially from higher plants and that 74% were found from ethnobotanical information. Certain areas of vegetation (Savannah and Guinean forest) are rich in species and types of environments to be used to search for natural product compounds, whose choice can be based on ethnobotanical and chemotaxonomic studies, and screen for their ability to inhibit activities. Globally, only a small proportion, out of the several thousand plant species has been investigated both phytochemically and pharmacologically, when one considers that a single plant may contain up to thousand of constituents, the possibilities of making new discoveries become evident (Hostettmann et al., 1995).

In 1993, during the world TB day, WHO declared TB as a 'global emergency' which requires emergency action and launched several programmes to attack the disease, including the search for newer remedies and/or anti-TB agents to complement currently used agents (WHO, 2002). Current epidemiological evidence suggests that TB is the most lethal infection worldwide, due to a single agent, *Mycobacterium tuberculosis*, even surpasses malaria. One-third of the world's population is infected with *M. tuberculosis*, 8 million new cases emerge annually and 3 million people are killed annually (Dye et al., 1999). In fact, TB still kills 5000 persons every day. The highest mortality is in the African region (WHO, 2004). The emergence of drug-resistant strains of *M. tuberculosis* has led to increased pressure on current chemotherapy regimens. The most advanced shorter TB treatment regimen presently in development could be available to the public by the end of 2009, if positive results continue (WHO, 2006). A shorter TB regimen will also help improve treatment adherence and preventing the development of multi drug-resistant TB. There is no doubt that the discovery of effective new agents are needed to deal with the current situation. In addressing the present situation, it was discovered that higher plant extracts are promising sources of novel anti-TB leads (Mitscher and Baker, 1998). Plants used in this study were selected based on the performance index ($I_p > 0$) of plants used to treat TB or related symptoms in Niger State, Nigeria (Mann et al., 2007). The aim of this study was to evaluate Nigerian medicinal plant extracts for activity against *M. tuberculosis* and *Mycobacterium bovis* (BCG) with the ultimate goal of identifying preparations with potential efficacy in the treatment of TB infections.

MATERIALS AND METHODS

Plant materials

An ethnobotanical survey of indigenous flora for the treatment of TB and other respiratory diseases in Niger State, Nigeria was conducted in order to select a group of plants used for the respiratory ailments (Mann et al., 2007). Based on the index of performance (I_p) values, eight plant species were selected. The plant parts used were obtained as described by traditional medical practitioners from a forest near Baddegi, Niger State, Nigeria. Voucher specimens were deposited in the herbarium at the National Institute for Pharmaceutical Research and Development (NIPRD). Approximately 3 kg of fresh plant material of each species was collected and air-dried.

Preliminary extraction of the plant materials

2 kg (1kg) of each plant material were powdered and extracted by percolation (cool maceration) for 72 h at room temperature with methanol (3 x 2.5 mL), that is, the methanol extracts were filtered and the process was repeated three times for exhaustive extraction, to ensure that no metabolites were left in the residues). All the extracts for each set were combined and then concentrated under low pressure to dryness at 35°C using a rotavapor. The dried methanol extracts obtained from each plant were air-dried then packed in glass bottles with proper labelling for future reference. The extracts were kept refrigerated and away from light (wrapping with aluminum foil prior to further processing).

Stock solutions were prepared in dimethyl sulphoxide (DMSO) at a concentration of 20 mg/mL and stored at -20°C until use. All the crude extracts of each plant species were tested for antimycobacterial activity.

Phytochemical analysis of extracts

The plant extracts were phytochemically screened using standard techniques for the detection of carbohydrates, saponins, tannins, terpenoids, glycosides and alkaloids (Brain and Turner, 1975; Harbone, 1975).

Mycobacterium species

M. tuberculosis clinical isolates was obtained from the Diagnostic Laboratory of National Institute for Pharmaceutical Research and Development (NIPRD), P.M.B.21, Garki – Abuja, Nigeria and the activity evaluation against *M. tuberculosis* was done at the TB Research Section, Zankli Medical Laboratory, Garki-Nigeria. BCG was obtained from National Institute of Allergic Diseases (NIAD), TB Research Section, NIH, Maryland, USA and cultured at Department of Microbiology and Biotechnology, National Institute for Pharmaceutical Research and Development (NIPRD), Garki – Abuja. BCG were grown on Lowenstein Jensen medium (LJ) and then subcultured in Middlebrook 7H9 broth supplemented with Albumin Dextrose Complex (ADC) at 37°C for 14 - 21 days.

Inocula preparation

The clinical isolate of *M. tuberculosis* and BCG cultured and grown on Lowenstein Jensen medium (LJ) and then subcultured in Middlebrook 7H9 broth supplemented with Albumin Dextrose Complex (ADC) at 37°C for 14 - 21 days were measured on spectrophotometer to be OD 0.2 - 0.3 at 650 nm. The cultures were then diluted at $1/1000$ by adding 25 μ L cell culture to 25 mL medium.

Antimycobacterial activity susceptibility test

The susceptibility test was conducted using the broth microdilution method (BMM) in 96 well microtitre plates. To wells No 2-12 of the 96-well microtitre plate was added fifty microlitres (50 μ L) of medium solution. 100 μ L of extract was pipetted into well No 1 from which 50 μ L was transferred to well 2. The content of well 2 was then mixed thoroughly and 50 μ L was transferred to well 3, repeated through to well 11 from where 50 μ L was discarded. Well 12 served as organism control.

Extracts were first dissolved in DMSO and then diluted in Middlebrook 7H9 broth, to give a stock concentration of 5000 μ g/mL which was diluted out across a 96-well microlitre plate in a two-fold serial dilution to give final testing concentrations of 2500, 1250, 625, 312, 156 and 78 μ g/mL.

The same procedure was repeated for the control (Rifampicin) with the initial concentration of 32 μ g/mL with the subsequent dilution to the final testing concentrations of 1, 0.5, 0.25, 0.125, 0.06, 0.03 μ g/mL. Appropriate DMSO, growth and sterile controls were carried out with rifampicin as positive control. Rifampicin control and negative control sets were triplicated for all the sets on each plate.

The plates were inoculated with the diluted culture for well 1-12. The plates were then incubated for 5-7 days at 37°C. After this incubation, any well before those that turned cloudy were recorded as the highest dilution of test compound that prevented bacterial growth (MIC). The minimum inhibitory concentration (MIC) was defined as the lowest extract concentration at which no mycobacterial growth was observed.

RESULTS AND DISCUSSION

Plants were selected based on the performance index ($I_p > 0$) of plants used to treat TB or related symptoms as reported in the survey (Table 1). Sequential extractions and isolation were done following the schematic procedures outlined in Figure 1. All extractants for each plant were separately combined and the resultant soluble fractions were concentrated and dried *in vacuo*.

The crude extracts were then separated using column and preparative, thin layer chromatographies on silica gel. The isolates were recovered from the silica gel as soon as the preparative plate TLC separation was complete in order to avoid reaction and formation of artifacts. The appropriate developing solvent system was determined by manipulating the ratios of the mixture of different solvents, mainly hexane, ethylacetate, methanol and acetic acid. The TLC separation yielded different fractions of compounds with different polarities.

Each fraction was tested for purity using TLC analysis with varying solvent systems. The process of Preparative Thin Layer chromatography (PTLC) separation and purification was repeated for each fraction isolated. Few drops of acetic acid were used to prevent tailing in the TLC separation.

Tuberculosis is the leading killer of youths, women, and AIDS patients worldwide. Its agent, *M. tuberculosis* causes more death than any other single infectious disease on earth. Search for new anti-tuberculosis drugs become obvious due to the above reasons and others such as development of multi-drug resistant TB, shortage

and expensive nature of TB drugs. Herbal remedies become the readily alternative in the search for new antimycobacterial compounds. The plants screened in this study were used in the traditional medicinal practices in Nigeria for the treatment of TB and other respiratory diseases (Mann et al., 2007). *Anogeissus leiocarpus*, *Terminalia avicennioides*, *Combretum* spp. and *C. brassii* had been reported as remedies for the management of tuberculosis (Mann et al., 2007). The crude methanolic extracts from the eight plants were separately percolated with methanol to give the extractive values in Table 2. The extractive values of the eight selected plant species ranged from 1.92- 20.62%. The details are shown in Table 2. The present study revealed that, six plants out of eight exhibited some degree of antimycobacterial activity against *M. tuberculosis* clinical isolate at ≤ 1250 μ g/mL. Only two plants namely *A. leiocarpus* and *T. avicennioides* showed strong inhibitory activity at 78 μ g/mL (Table 2). While *Combretum* spp. and *C. brassii* showed activity against *M. tuberculosis* at 1250 μ g/mL. Uba et al. (2003 a,b) and Johnbull and Abdu (2006) also found *A. leiocarpus* active. Jimenez-Arellanes and co-researchers earlier reported the antimycobacterial activity of the hexane extracts of Mexican plants used to treat respiratory diseases (Jimenez-Arellanes et al., 2003).

These results justify the traditional use of these plants for the treatment of TB or its symptoms as shown in Table 1 and also substantiate their potentials as sources of leads for the development of active antituberculosis agents. However, the crude extracts of *E. africana*, *H. acida* and *S. kunthianum* tested did not exhibit any significant anti-tuberculosis activity, even at concentration as high as 1250 μ g/mL. Although these plants are traditionally used for treating TB and other respiratory diseases, it is possible that these plant extracts are effective against ailments such as bronchitis, cough and asthma caused by agents other than *Mycobacteria*. Screening directly against tuberculosis agent is hazardous because of the highly infectious nature of the pathogen and is also tedious because the organism is comparatively slow growing (Bloom, 1994). Extracts were prepared and screened initially with *M. tuberculosis* and subsequently the attenuated strain of *M. bovis* (BCG) was used as a surrogate test organism. Since BCG, just like *M. smegmatis* is sensitive *in vitro* to the action of many established antitubercular, anti-infective grows fairly rapidly and does not normally infect healthy humans (Mitscher and Baker, 1998).

The results of the phytochemical analysis (Table 3) prominently indicate the presence of saponins, steroids, tannins, terpenes, anthraquinone and carbohydrates. The extractive values of the two active plants for hexane extracts ranged from 0.30 - 0.32%, ethyl acetate extracts from 6.22 - 8.56%, and methanol extracts from 4.8 - 15.36%. The details are shown in Table 4.

The crude extracts of the two active plants were further fractionated and evaluated to determine which solvent fractions contain the most active constituents. Therefore,

Table 1. Ethnobotanical data of eight selected Nigerian medicinal plants used in this study

| Voucher | Scientific names (Family) | Common names ^a | LGA ^b | Plant Part ^c | Folkloric Usage ^d | References |
|-------------|--|---|--|-------------------------|--|--|
| ABUHH 167 | <i>Anogeissus leiocarpus</i> (DC.) Guill and Perr. (Combretaceae). | Marike (H) Shici (N) Ayin (Y) Suasuan veyi(G) | Bida, Gbako, Mariga, Paikoro, Patigi | Sb Rb | Asthma, Cough Tuberculosis, Worm killer, gonorrhoea | Mann et al., 2007; Etkin, 1981. |
| NIPRDH 5750 | <i>Capparis brassii</i> DC (Capparidaceae) | Ekanci-wuriangi (N) | Gbako | Rb | Tuberculosis | Mann et al., 2007 |
| NIPRDH 5750 | Combretum Spp (Combretaceae) | Kukunci (N) | Bida, Bosso, Kontagora, Lavun, Minna | Sb Rb | Bronchitis, Cough, Tuberculosis | Mann et al., 2007 |
| NIPRDH 5739 | <i>Entada africana</i> Guill & Perr. (Mimosaceae). | Tawatsa (H) Locigaga (N) Bwadari (G) Ogurube (Y) | Agaie, Bosso, Lavun, Mariga, Minna, Paikoro | Sb Rb | Bronchitis, Cough, Whooping cough Dysentery, Fever, wound | Gill, 1992; Mann et al., 2003, 2007 |
| NIPRDH 5741 | <i>Hymenocardia acida</i> Tul. (Euphorbiaceae) | Janyaro (H) Lukpa (N) | Lavun, Mariga, Paikoro, Rijau | Rb | Hemoptysis, Tuberculosis | Mann et al., 2007 |
| ABUH 900252 | <i>Sterculia setigera</i> Del Sterculiaceae | Kukuki (H) Bokoci (N) Esofunfun (Y) Mumuyi (G) | Bosso, Gbako, Mariga, Minna, Mokwa | Sb | Asthma, Bronchitis, Cough, wound, boils Hemoptysis Fever, Cancer, dysentery, | Mann et al., 2003, 2007 |
| ABUH 1381 | <i>Stereospermum kunthianum</i> Cham. (Bignoniaceae) | Jiri (H) Ayada (Y) Dagbapanoci(N) Kushishigban(G) | Lavun, Mariga, Minna | Sb | Cancer, Tuberculosis | Mann et al., 2003, 2007 |
| NIPRDH 5735 | <i>Terminalia avicennioides</i> Guill. & Perr. Combretaceae | Baushe (H) Edo (I) Kpace(N) Igiotan (Y) Kpayi (G) | Agaie, Bosso, Kontagora, Lavun, Mokwa, Paikoro, Patigi | Mt Rb Fr | Asthma, Cough, Hemoptysis, Tuberculosis, Sore throat, diarrhea | Mann et al., 2003, 2007 |

^aLocal names commonly used by the people: G- Gwari, H- Hausa, I- Igbo, N- Nupe and Y- Yoruba,

^bLocal government Area of folkloric use in Niger state, Nigeria.

^cPlant part used: F = Fruit, Mt = Mistletoes, Rb = Root bark, Sb = Stem bark.

^dFolkloric uses.

the fractionation of the active crude methanol extracts led to further investigation of the *n*-hexane, ethyl acetate and methanol extracts. The *n*-hexane and ethyl acetate extracts of *A. leiocarpus* and *T. avicennioides* showed the most significant inhibitory activity (312.5-625 µg/mL) (Table 4). Both *n*-hexane and ethyl acetate extracts of the two plants (Al and Ta) were then subjected to silica gel and sephadex chromatographies and the antimycobacterial activity were then conducted on the extracted fractions against BCG (Figure 1). Most importantly, the activity showed by the hexane extract of both *A. leiocarpus* and *T. avicennioides* against BCG deserves special attention since the fractions were active at low

concentrations of 312 and 200 µg/mL respectively. These results are in conformity with the similar results obtained for crude plant extracts to be considered active (Frame et al., 1998; Houghton et al., 1999).

The compounds investigated for activity against BCG were Ta and Al. The results are summarized in Table 5. The hexane fractions of the two plants contain terpenoids and saponins, which may be responsible for the associated activity. Compounds Ta4 and Alfa showed weak activity against BCG. Compared to the positive control, rifampicin (MIC 0.4 µg/mL), Ta5 and Al4 exhibited moderate activity (MIC 4.7 and 7.8 µg/mL respectively). Compound Ta5a possessed no activity against BCG.

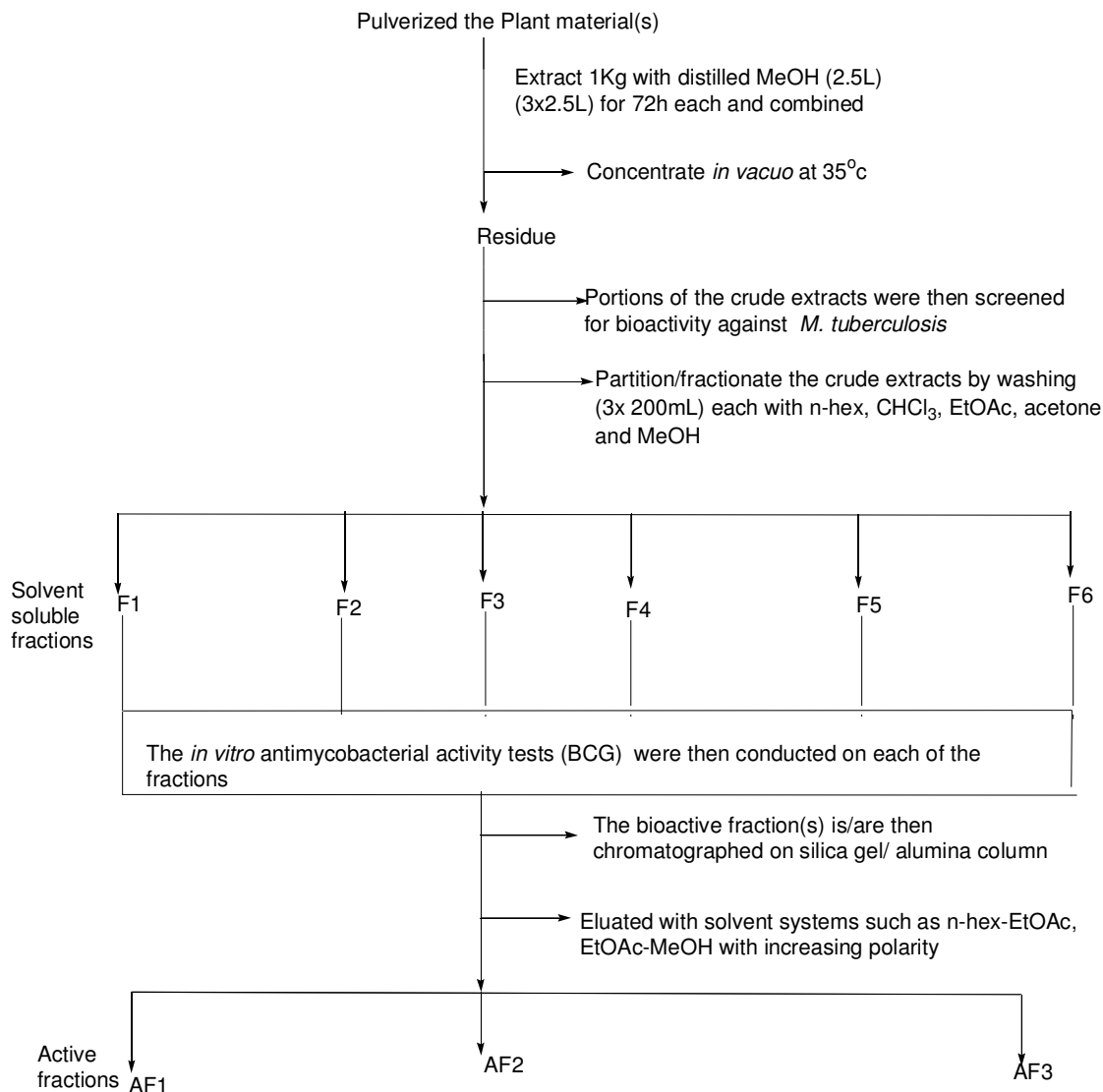


Figure 1. Partitioning and fractionation of plant material with antimycobacterial activity.

Table 2. The extractive values and Antimycobacterial activity of crude methanol extracts against isolated *Mycobacterium tuberculosis* culture

| Plant | Amount extracted (g) per 100 g crude ^a | Activity at 2500 µg/mL ^b | MIC (µg/mL) ^c |
|---|--|--|--------------------------|
| <i>Anogeissus leiocarpus</i> (DC.) Guill. & Perr. | 8.8 | Active | 78 |
| <i>Capparis brassii</i> DC | 3.15 | Active | 1250 |
| <i>Combretum spp</i> | 7.3 | Active | 1250 |
| <i>Entada africana</i> Guill. & Perr. | 5.44 | Active | 2500 |
| <i>Hymenocardia acida</i> Tul. | 6.96 | Inactive | >2500 |
| <i>Sterculia setigera</i> Del. | 4.04 | Active | 2500 |
| <i>Stereospermum kunthianum</i> Cham. | 1.92 | Inactive | >2500 |
| <i>Terminalia avicennioides</i> Guill. & Perr. | 20.62 | Active | 78 |
| Ethambutol | | | 2 |

^aExtractive values.

^bActivity at 2500 µg/mL.

^cMinimum Inhibitory Concentration.

Table 3. Phytochemical screening results of the crude methanol extracts

| Plant species | A | An | Cb | F | S | St | T | Tp |
|---|---|----|----|---|---|----|---|----|
| <i>Anogeissus leiocarpus</i> (DC.) Guill. & Perr. | - | + | + | - | + | + | + | + |
| <i>Terminalia avicennioides</i> Guill. & Perr. | - | + | - | - | + | + | + | + |

+ = present, - = absent, A = Alkaloids, An = Anthraquinone, Cb = Carbohydrate, F = Flavonoid, S = Saponin, St = Steroids, T = Tannin, Tp = Terpenoids.

Table 4. Antimycobacterial activity of the extracts against BCG.

| Plant ^a | Amount extracted (g) per 100g ^b | Activity at 1250 µg/mL ^c | MIC (µg/mL) ^d |
|--------------------|--|-------------------------------------|--------------------------|
| AlHs. | 0.32 | Active | 312.5 |
| AlEs. | 6.22 | Active | 625 |
| AlMs. | 4.8 | Inactive | >1250 |
| TaHs | 0.30 | Active | 200 |
| TaEs | 8.56 | Active | 625 |
| TaMs | 15.36 | Inactive | >1250 |
| Rifampicin | | | 0.03 |

^aPlant solvent soluble fraction.

^bExtractive values.

^cActivity at 1250 µg/mL.

^dMinimum Inhibitory Concentration.

Es = Ethylacetate soluble, Hs = hexane soluble, Ms = Methanol soluble, Al = *Anogeissus leiocarpus*, BCG = Bacillus Calmette Guerin, Ta = *Terminalia avicennioides*.

Table 5. Antimycobacterial activity of the Al and Ta fractions from fcc against BCG

| Isolated compound | MIC (µg/mL) |
|-------------------|-------------|
| Al4 | 7.8 |
| Alfa | 12.5 |
| Ta4 | 12.5 |
| Ta5 | 4.7 |
| Ta5a | NA |
| Rifampicin | 0.4 |

Al = *Anogeissus leiocarpus* fraction, BCG = Bacillus Calmette Guerin, fcc = flask column chromatography, A = Not Active, Ta = *Terminalia avicennioides* fraction.

Both compounds (Ta5 and Al4) from the phytochemical analysis were terpenoid derivatives, suggesting that this group of terpenoidal moiety might be important for the observed activity.

Copp (2003) reviewed antimycobacterial natural products where some metabolites such as terpenes (monoterpenoids, diterpenes, sesquiterpenes and triterpenes), steroids and alkaloids found to be abundant in many extracts of the studied plants were reported to possess potential structural skeletons that could provide useful scaffolds or templates for the development of new

antimycobacterial drugs. Many plants have been used through the ages for the therapy of TB and its symptom. It is not improbable that among them a real remedy exists. This observation makes this class of compounds with high activity interesting for further investigation and special attention on the Combretaceae family from Nigeria flora, of which *A. leiocarpus* and *T. avicennioides* are members. The present study demonstrates that Nigerian medicinal plants could be good sources of compounds with antimycobacterial activities worthy of investigation. Further investigations, particularly the chromatographic purification of the most active fractions of the two plants are currently being conducted to isolate the active compounds.

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