



## Phytochemical constituents and anti-tuberculosis activity of leaf extracts of *Berlinia grandiflora* (Vahl) Hutch. & Dalziel

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### Abstract

Tuberculosis (TB) remains one the most significant infectious diseases of public health importance in Nigeria. This study is aimed at analyzing the phytochemical constituents and anti-tuberculosis activity of leave extracts of *Berlinia grandiflora*. Successive exhaustive extraction of 100 g of the dried leaves of *Berlinia grandiflora* was done with the aid of a soxhlet extractor using n-hexane, ethyl acetate and methanol respectively. The aqueous extract was prepared by infusion of 100 g of another portion of the dried leaves of the plant for 24 hours. *Mycobacterium bovis* (27290) obtained from Medimark, France was grown and sub-cultured in Middle brook 7H9 broth supplemented with Albumin Dextrose Complex (ADC) at 37°C for 14-21 days. The effects of various extracts of *Berlinia grandiflora* were determined against *M bovis*, while the Minimum Inhibitory Concentrations (MICs) were evaluated using standard techniques. The result from this study reveals that the aqueous extract contains carbohydrates, terpenoids, steroids, alkaloids, flavonoids, saponins, phenols and tannins, while proteins and anthraquinone were absent. The methanol extract contains alkaloids, flavonoids, tannins, carbohydrates, phenols and saponins while protein, terpenoids, steroids, anthraquinone were absent. For the ethyl-acetate extract, the result reveals the presence of carbohydrates, alkaloids, flavonoids, terpenoids, phenols and tannins but showed a negative result to protein, saponins, phenols, steroids and anthraquinone. The hexane extract contain only terpenoids and steroid. Results from this study further showed that n-hexane and ethyl-acetate were the most effective extract in inhibiting the growth of *M. bovis* with an MIC of 3.9 µg/mL while the methanol and aqueous extract inhibited the growth with MICs of 7.8 µg/mL and 31.2 µg/mL respectively. The anti-tuberculosis activity exhibited by the extracts of *Berlinia grandiflora* justifies its folklore use and also shows the potential of the plant in TB management.

**Keywords:** Tuberculosis, *Mycobacterium bovis*, Minimum Inhibitory Concentration, *Berlinia grandiflora*

### Introduction

Tuberculosis remains one the most significant infectious diseases of public health importance in Nigeria. It is estimated that about 1 billion people are infected with TB, with about 10 million new cases and 3 million deaths per

annum, worldwide. In 2010, Africa had a TB incidence of 2.3 million and a prevalence of 2.8 million with about 250,000 recorded deaths (WHO, 2010). Furthermore, Nigeria is

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ranked seventh in the world and second in Africa among the 30 countries with the highest burden of TB (WHO, 2017). In 2015 six countries Nigeria, China, India, South Africa, Pakistan and Indonesia accounted for 60% of total TB cases worldwide. Despite a 1.5% reduction in the incidence of TB between 2014 and 2015, the increase in new cases of multidrug resistant tuberculosis has made TB treatment even more difficult. About, 480,000 new cases of multi-drug resistant TB were recorded in 2015 (WHO, 2016).

Almost all the species of *Berlinia* is found in Central and West Africa with about twenty (20) species which are limited to tropical Africa. *Berlinia grandiflora* is widely distributed in Guinea, Mali, Nigeria, Central Africa and Democratic Republic of Congo (Hutchison and Dalziel, 1963). *Berlinia grandiflora* (Leguminosae) *Caesalpinioideae* is a medicinal plant, which could be a potential anti TB agent. Locally in Nigeria, it is called Apado by the Yoruba people and Ububa by the Igbo ethnic groups (Joseph *et al.*, 2012). *Berlinia grandifolia* is well known in among herbalist in the Nigerian traditional medicine practice for managing several disease conditions such as skin infections, haemorrhoids and liver complications (Joseph *et al.*, 2012), cancer (Mike *et al.*, 2010), vermifuge, diarrhea and Diabetes (Ode *et al.*, 2013). Folks use the leafy twigs decoction as febrifuge, cholagogue, purgative and anti emetic, while the leaf decoction is also drunk as tonic (Mackinder and Harris, 2006).

Joseph *et al.*, 2012 reported that the leaf and stem of the plant is used by herbalists in South Eastern Nigeria, to treat microbial infections. Sap from its bark is used for treatment of sores and wounds, while bark decoctions are administered for treatment of haemorrhoids and liver complaints (Gill, 1992). The bark is also used to ease labour during child birth and gastrointestinal disorders.

Although there is paucity of scientific information on the biological activity of the plant, the analgesic activity of stem bark extract (Asuzu *et al.*, 1993), antihelminthic activity of stem bark and its active principle, betulinic acid (Enwerem *et al.*, 2001) have been reported.

However, this ethno medicinal use of the plant parts has not been validated scientifically. Although a number of anti tuberculosis drugs already exist, the emergence of multidrug resistance strains coupled with long treatment duration and synergy with HIV have necessitated the search for new, more effective and more user friendly anti TB drugs from natural sources like *Berlinia grandiflora*. Thus, the aim of this study was to evaluate the anti-tuberculosis activity of the n-hexane, ethyl-acetate, methanol and aqueous extracts of *Berlinia grandiflora* leaves against *M. bovis*.

## Materials and Methods

### Sampling and Sample preparation

Fresh leaves of *Berlinia grandiflora* were collected from Chaza, Suleja local government, Niger state, Nigeria and was taxonomically identified with a voucher number of NIPRD/H/6966 at the Herbarium unit, National Institute for Pharmaceutical Research and Development (NIPRD). The plant was air dried at room temperature for about 10 days, pulverized and stored in a well labelled container for further analysis.

### Extraction of bioactive constituent

Bioactive compounds were extracted from 100 g of the plant sample with the aid of a soxhlet apparatus. The powdered samples were successively extracted starting with n-hexane, followed by ethyl-acetate, and then methanol and water. The n-hexane, ethyl-acetate and methanol extracts were concentrated using the rotary evaporator while the water extract was concentrated on the water bath. The extracts were

stored in a well labelled sample bottle and kept in desiccators for further analysis.

**Phytochemical analysis:** Some portion of the extracts was reconstituted in the same solvent used for extraction, the following test were done on the extract (Evans, 2004; Sofowora, 1993; Trease and Evans, 1989).

#### **Test for carbohydrate**

**Molisch's Test:** To the dissolved extracts, 3-4 drops of Molisch's reagent was added, followed by small quantity of concentrated Sulphuric acid and allowed to form layer. The formation of a purple ring at the interface indicates the presence of carbohydrates.

#### **Test for alkaloids**

**Wagner's Test:** The extracts were treated with drops of Wagner's reagent (Iodine in Potassium Iodide). The formation of brown/reddish precipitate shows the presence of alkaloids.

**Dragendorff's Test:** On addition of drops of Dragendorff's reagent (potassium bismuth Iodine solution), the formation of precipitate or turbidity reveals the presence of alkaloids in the extracts.

**Hager's test:** Few drops of Hager's reagent (solution of picric acid) was added and formation of yellow solution indicated the presence of alkaloids.

#### **Test for saponins**

**Froth Test:** Two (2) mL of distilled water was added to 0.5 gm of the extracts and vigorously shaken until foam is formed. If the foam produced persists for ten minutes it indicates the presence of saponins.

#### **Test for phenols/tannins**

**Ferric Chloride Test:** Three to four (3-4) drops of 10% ferric chloride solution was added to the extracts. Formation of bluish black colour indicates the presence of phenols.

#### **Test for flavonoids**

**Sodium Hydroxide Test:** Add 2 mL of 10% Sodium Hydroxide solution to 2 mL of the extracts. A yellow solution indicates the presence of Flavonoids.

**Ferric Chloride Test:** Add drops of 10% ferric chloride solution to the extracts. Formation of dark green colour precipitate indicates the presence of flavonoids.

**Shinoda Test:** Four (4) pieces of Magnesium filings was added to the extracts, followed by few drops of Concentrated Hydrochloric acid. A pink or red color indicates the presence of Flavonoids.

#### **Test for proteins**

**Million's test:** The dissolved extracts were treated with drops million's reagent. Formation of white precipitate which turned red upon gentle heating showed the presence of proteins.

#### **Test for anthraquinones**

**Borntrager's test:** Add 5 mL of chloroform to 2 mL of extracts; shake the mixture for about 5 minutes. Add 2 mL 10% ammonia solution to 2 mL of the extracts. A pink-violet or red colour in the ammonia layer (lower layer) indicates the presence of free anthraquinones.

#### **Test for terpenoids**

**Salkowski test:** Two (2) mL of the extracts were treated with 2 mL of chloroform and 3 mL of concentrated sulphuric acid, to form a layer. A reddish brown colouration of the interface confirms the presence of terpenoids.

#### **Test for steroids**

**Lieberman-Burchard test:** Add 2 mL of acetic anhydride to 2 mL of the extracts followed by 2 mL of H<sub>2</sub>SO<sub>4</sub>. Colour change from violet to blue, or green in indicate the presence of steroids.

### Culture and growth of mycobacteria

*Mycobacterium bovis* (27290) was obtained from Medimark, France and cultured at the Department of Microbiology and Biotechnology, National Institute for Pharmaceutical Research and Development (NIPRD), Abuja. The organism was grown and then sub-cultured in Middle brook 7H9 broth supplemented with Albumin Dextrose Complex (ADC) at 37°C for 14 – 21 days.

### Preparation of Inoculum

Fifty micro-litre of freshly thawed stock test organism (*M. Bovis*) was inoculated into 50 mL of sterile Middle brook 7H9/ADC media and incubated at 37°C with shaking for 5-7 days. The activity grown *M. bovis* culture had its optical density adjusted to between 0.2 - 0.3 at a wavelength of 650 nm using Jenway 6405 UV-Visible spectrophotometer.

### Determination of Minimum Inhibitory Concentration (MIC)

The susceptibility test was conducted using the broth micro-dilution method in 96 well micro-titre plates (Wayne, 2009). Extracts were first dissolved in DMSO and then diluted in Middle brook 7H9 broth, to give a starting concentration of 1000 µg/mL which was diluted across the 96-well micro-litre plate in a two-fold serial dilution to give final testing concentrations of 500 µg/mL to 3.9 µg/mL. Isoniazid which was dissolved in DMSO and used as positive control drug and extracts/drug free medium with culture suspensions were used as negative control. Each extract concentration was assayed in duplicate. The plates were then incubated for 5-7 days at 37°C. After the 7<sup>th</sup> day, 25 µL of tetrazolium salt dye was added to all the wells, re-incubated over-night and observed for

absence or presence of microbial growth by colour change in the wells. The MIC was defined as the lowest drug/extract concentration that prevented the color change of the tetrazolium dye to pink. Colourless well was interpreted as there is no mycobacterial growth and pink color was interpreted as growth occurrence.

### Result

In this study, the presence of carbohydrate, protein, phenolics, tannins, flavonoids, alkaloid, saponins, terpenoids, steroids and anthraquinone in the extracts was investigated. The result of the phytochemical screening shows that the aqueous extract contains more phytochemicals than the other extracts. All the phytochemicals investigated in this study were detected in the aqueous extract except, proteins and anthraquinone (Table 1). The methanol and ethyl-acetate extracts contains all the phytochemicals investigated, except protein, terpenoids, steroids, anthraquinone which were absent in the methanol while, protein, saponins, phenols, steroids, saponin and anthraquinone were not detected in the ethyl-acetate extract. The hexane extract contain fewer numbers of phytochemicals as, only terpenoids and steroid were detected (Table 1). In term of number of phytochemicals in extract, the following pattern has been established from the result of this study; hexane <ethyl-acetate< methanol<water. From Table 2 below, the n-hexane and ethyl-acetate extracts were the most effective in inhibiting the growth of *M. bovis* with an MIC of 3.9µg/mL while the methanol and aqueous extracts are less effective with MICs of 7.8 µg/mL and 31.2 µg/mL respectively.

**Table 1:** Phytochemical analysis of aqueous, methanol, ethyl-acetate and n-hexane extracts of *Berlinia grandiflora* leaves

Phytochemicals	Test	Water	Methanol	Ethyl-acetate	N-Hexane
Carbohydrate	Molisch Test	+	+	+	-
Terpenoids	Salkowski's Test	+	-	+	+
Steroids	Liebermann-burchard	+	-	-	+
Anthraquinone	Borntrager's Test	-	-	-	-
Alkaloids	Wagner's Test	+	+	+	-
	Dragendoff's Test	+	+	+	-
	Hager's Test	+	+	+	-
Flavonoids	Sodium Hydroxide	+	+	+	-
	Ferric Chloride	+	+	+	-
	Shinoda	+	+	+	-
Tannins	10% Ferric Chloride	+	+	+	-
Phenols	10% Ferric Chloride	+	+	+	-
Proteins	Million's	-	-	-	-
Saponins	Froth Test	+	+	-	-

Key: (+) present, (-) absent.

**Table 2:** Minimum Inhibitory Concentration (MIC) Values of N-Hexane, Ethyl-acetate, Methanol and Aqueous extracts of *Berlinia grandiflora* leaves against *M. bovis*

Minimum Inhibitory Concentration Values ( $\mu\text{g/mL}$ )					
Organism	N-Hexane	Ethyl-acetate	Aqueous	Methanol	Isoniazid
<i>M. bovis</i> (27290)	3.9	3.9	7.8	31.2	0.04

## Discussion

The increase in multidrug resistance TB has led researchers to constantly search for new antimicrobials that could help fight against this scourge. There is also urgent need of finding newer anti-tuberculosis agents to fight this problem due to increase rate in MDR and XDR strains of *M. tuberculosis*.

Results of this study show that the extracts of *Berlinia grandiflora* contain varied number of phytochemicals which have been reported to possess antimicrobial properties against several bacterial strains. Previous studies have revealed that *Berlinia grandiflora* contains tannins, alkaloids, flavonoids, glycosides, saponins, phenols.

However, anthraquinone and carbohydrate were absent according to (Ode *et al.*, 2013). These phytochemicals are known to be bactericidal, pesticidal or fungicidal in nature (Lutterodt *et al.*, 2005). The result of the phytochemical analysis in this study is consistent with the results obtained by Asuzu *et al.*, (1993) and Duru *et al.*, (2014). The presence of a plethora of phytochemicals found in *Berlinia grandiflora* as seen in this study, is a testament to the antimicrobial activities and medicinal values of the plant, since these phytochemicals confers therapeutic properties on plants. Studies have shown that these phytochemicals are responsible for various therapeutic activities of medicinal plants. Tannins have been found to form irreversible complexes with proline proteins resulting in the inhibition of cell protein synthesis. This is part of the mechanism for antimicrobial activity of plant (Scalbert, 1991). They are also potent anti-oxidants and also used for the treatment of diarrhea and dysentery (Cushnie, 2005). Flavonoids acts as 'biological response modifiers' such as anti-allergic, anti-inflammatory, anti-microbial and even the in vitro studies show it has anti-cancer activities (Rice-evans *et al.*, 1995). These activities can be attributed to their ability to form complex with extracellular and soluble proteins with microbial cell wall. Saponins are very good in reducing the blood cholesterol level, it can inhibit the growth of cancerous cells, stimulation of the immune system and even bone health (Trease and Evans, 1989). Steroids are good in treating bone-degenerative diseases.

Li *et al.*, (2015) reported that four pentacyclic lupine triterpenes; betulin, betulinic acid, betulone and lupenone which were isolated form methanol extract of the bark of *Alnusincana* possess anti-mycobacterial activities against *M. tuberculosis* (H37Ra). Betulin have an MIC value of 12.5 µg/mL and IC50 value of 2.4 µg/mL, while, betulinic acid and

betulone possesses lower anti-mycobacterial activities with IC50 of 84 and 57 µg/mL respectively. In addition, Morrison *et al.*, (2016) reported that four triterpenes; betulinaldehyde, β-sitosterol, betulinic acid, and ursolic acid which were isolated from methanolic extracts of *Sarracenia purpurea* showed ant-imycobacterial activity against H37Ra *Mycobacterium tuberculosis* strain. The MIC for betulinaldehyde, betulinic acid, and ursolic acid are 450, 950, and 450 µM and the IC50s are 98, 169, and 93 µM respectively. The author identified betulinaldehyde, betulinic acid, and ursolic acid as the principal constituents responsible for the anti-mycobacterial activity of *S. purpurea*. Akuodor *et al.*, (2011) reported that methanolic extract of *B. grandiflora* have concentration dependent inhibitory effect on *E. coli*, *S. aureus* and *Streptococcus* spp.

In a previous study, Enweren *et al.*, (2001), isolated betulinic acid, a terpenoid from *Berlinia grandiflora* and attributed the antimicrobial activity of *Berlinia grandiflora* to the betulinic acid. Similarly, the anti-mycobacterial activity of *Berlinia grandiflora* in this study could be attributed to the presence of betulinic acid, its derivatives, other terpenoids and the synergetic effect of other phytochemicals, since n-hexane and ethyl acetate extract of *B. grandiflora* which are the most effective against *M. bovis* both contain terpenoids.

### Conclusion

The anti-tuberculosis activity exhibited by *Berlinia grandiflora* could be attributed to the presence of terpenoids in the plant. Furthermore, this work justifies the folklore use of *Berlinia grandiflora*. It is also a pointer to the fact that the plant can be harnessed for their potential anti-tuberculosis therapy and developed into anti TB phytomedicine. This could help reduce the overall burden of the disease in Nigeria.

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