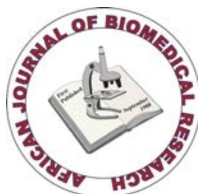
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Research article

In-Vitro Susceptibility of *Mycobacterium Tuberculosis* to Extracts of *Uvaria Afzelli* Scott Elliot and *Tetracera Alnifolia* Willd

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ABSTRACT: Tuberculosis is a global burden with one –third of the world's population infected with the pathogen *Mycobacterium tuberculosis* and an annual 2 million deaths from the disease. This high incidence of infection and the increased rate of resistant strains of the organism (MDR- and XDR- TB) have called for an urgent need to develop new anti-tuberculosis drugs from plants. The crude extract of *Uvaria afzelli* Scott Elliot (Annonaceae) root bark, and leaves and root bark of *Tetracera alnifolia* Willd. (Dilleniaceae) were investigated for anti-*Mycobacterium tuberculosis* activity using the MABA assay method. Anti- *Mtb* activity was determined against *Mtb* H37RvATCC 27294 at concentrations of 100-0.390µg/mL. The hexane and chloroform extracts of the root bark of *Tetracera alnifolia* and the chloroform extract of *Uvaria afzelli* had anti- *Mtb* activity with MIC <100 µg/mL. Phytochemical screening for secondary metabolites revealed the presence of tannins, triterpenoid saponins, cardiac glycoside and alkaloids. The anti- *Mtb* activity demonstrated by the crude extracts is attributed to the presence of tannins and other secondary metabolites which are known to have strong antimicrobial activity. The results therefore support the local use of *Uvaria afzelli* and *Tetracera alnifolia* in the treatment of cough associated with tuberculosis and other microbial infections of the respiratory tract and suggest that these plants may be of therapeutic importance in the treatment of tuberculosis.

Keywords: *Uvaria afzelli, Tetracera alnifolia*, crude extracts, *Mycobacterium tuberculosis*

INTRODUCTION

Infection with *Mycobacterium tuberculosis* (*Mtb*) results in tuberculosis (TB), a contagious disease with high mortality worldwide. About 2 billion people (or one-third of the world's population), are infected with *Mycobacterium tuberculosis.* Globally, there were an estimated 13.7 million chronic active cases, 9.3 million new cases and 1.8 million deaths mostly in developing countries in 2007 (WHO, 2009). It is estimated that there are currently 2 million deaths from tuberculosis annually (Deng *et al*., 2008). Moreover, up to 50 million people are infected with drug-resistant forms of TB with about 500,000 cases of MDR tuberculosis a year worldwide (WHO, 2007). The recent increase in the widespread existence of extensively drug-resistant tuberculosis (XDR-TB) especially in the developing nations emphasized the need for the development of new drugs to treat this infection. Such new anti-tubercular agents should have novel modes of action and full activity on the pathogen- *Mycobacterium tuberculosis.*

The use of herbs and other alternative therapies for the treatment of tuberculosis is on the increase. Natural products continue to play a most significant role in the drug discovery and development process (Newman and

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Cragg, 2007), and plants are recognized as a useful source of highly active antimycobacterial metabolites (Gibbons, 2005; Pauli *et al*., 2005).

The plant *Uvaria afzelli* belong to the family Annonaceae which contains 130 genera and about 2300 species, found mainly in the tropical regions of the old world (Trease and Evans, 1989). They are known to have compounds with pharmacological, insecticidal, antiprotozoal and antimicrobial activities (Kihampa *et al.*, 2009). The genus *Uvaria* includes approximately 150 species widely distributed in tropical areas particularly in Africa (Akendengue *et al.*, 2003). All parts of the plant are fragrant and are used to make pomade in Ghana (lrvine, 1961). *Uvaria* species are used locally in the treatment of various infections and diseases. The decoction of the leaves is used in the treatment of swollen face, hands and feet. A paste made from the ground leaves is rubbed over ulcers and wounds. A decoction of the fruits is used in the treatment of vaginal tumor and breast aches. The decoction of the whole plant is used in the treatment of leucorrhoea and gonorrhoea (Verger, 1995). The pulped leaves are eaten with oil palm seed for cough treatment (Kerharo and Bouquet, 1950). A decoction of the plant is used to wash a person with small-pox (Bouquet, 1974). *Uvaria afzelli* has been reported to have anti-parasitic activity (Okpekon *et al.*, 2004). It is used locally in the treatment of fever and cough (Kayode *et al.*, 2009). The root bark of *U. afzelii* is used in Nigeria for infections of the kidneys, bladders and liver and also a purge, a febrifuge and for coughs (Oliver, 1960).

The family Dillenaceae is almost pantropical with about 18 genera and 530 species. *Tetracera alnifolia* and other species of *Tetracera* have been reported to contain chemical constituents used in the treatment of various diseases and infections. A leaf decoction is taken for dysentery (Walker and Sillans, 1961). The roots of some *Tetracera* are used for yaws. A root macerate is used for urethral discharge (Kerharo and Bouquet, 1950). The plants lianous stems are macerated in its sap and taken for leprosy (Kerharo and Adam, 1962). The sap of *T. potararia* is used for toothache and cough (Oliver, 1960).

Flavonoids and coumarin derivatives are the main chemical constituents isolated from these plants genera (Akendengue *et al*., 2003; Hufford *et al*., 1981; Gurni *et al*., 1981). There are very few reports on the phytochemical and antimicrobial investigations on these plants in the literature. It is therefore the aim of this study to investigate the local use of these plants for the treatment of cough associated with tuberculosis and other respiratory tract infections.

MATERIALS AND METHODS

Plant collection and authentication

The root of *Uvaria afzelii* and the leaves and root of *Tetracera alnifolia* were collected. The samples were authenticated at the Herbarium, Forestry Research Institute of Nigeria (FRIN) and were assigned voucher specimen numbers FHI 107510 and 107511 respectively. The samples were air-dried, ground to coarse powder and weighed.

Plant extraction and preparation of extracts

Four hundred grams (400g) of *Uvaria afzelii* root bark, 300g leaf and 420g root bark of *Tetracera alnifolia* were subjected to exhaustive Soxhlet extraction with n-hexane and chloroform. The different fractions were concentrated in-vacuo, weighed and stored at room temperature until needed for assay. Stock solutions of each of the crude extracts were reconstituted with DMSO (1%) to final concentrations of 100µg/mL-0.390µg/mL for anti-*Mtb* assay.

Bacterial strain for anti-Mtb biological assays

Mycobacterium tuberculosis H37Rv ATCC 27294 was grown in 100 mL of Middlebrook 7H9 broth (Difco, Detroit, Mich.) supplemented with 0.2% (v/v) glycerol (Sigma Chemical Co., Saint Louis, Mo.), 10% (v/v) OADC (oleic acid, albumin, dextrose, catalase; Difco), and 0.05% (v/v) Tween 80 (Sigma), also referred to as 7H9GC-T80.

Susceptibility Testing of Mtb H37Rv ATCC 27294 and determination of minimum inhibitory concentration (MIC)

Anti-*Mtb* susceptibility testing of extracts was determined by the fluorometric Microplate Alamar Blue Assay (MABA) assay as described by (Collins *et al.*, 1997) in black, clear-bottomed, 96-well microplates, (black view plates; Packard Instrument Company, Meriden, Conn.) in order to minimize background fluorescence. Extracts were prepared to100µg/mL in DMSO (200µL) and subsequent twofold dilutions were performed in 100µL of 7H12 medium (i.e. 7H9 broth containing 0.1% ^{W}/v casitone, 5.6µg/mL palmitic acid, 5mg/mL bovine serum albumin, 4mg/mL catalase, filter-sterilized) in the microplates. The test concentrations ranged between 100µg/mL – 0.390µg/mL. Rifampin (RMP, Sigma; concentrations at 4-0.0156µg/mL) was used as a positive control. *Mycobacterium tuberculosis* H37Rv ATCC 27294 strain was diluted in 7H12 media to reach approximately 2×10^5 CFU/mL, and 100µL was added to individual wells containing the extracts yielding a final volume of 200µL and final inoculum of

$1x10^5$CFU/mL in each well. Wells containing extracts only were used to detect auto-fluorescence of extracts. Additional control wells consisted of bacteria only and medium only. Plates were incubated aerobically at 37 °C. At day 7 of the incubation, 20µL of Alamar Blue solution (Trek Diagnostic Systems, Cleveland, Ohio) and 12.5 µL of 20% Tween 80 were added to all of the wells, and the plates were re-incubated at 37 °C for 24 hours. Fluorescence was measured in a Victor III multilabel fluorometer (Perkin Elmer Life Sciences Inc., Boston, MA) in bottom-reading mode with excitation at 530 nm and emission at 590 nm. Anti- *Mtb* activity was recorded as percentage inhibition of ≥90% relative to the mean of replicate bacteria-only controls after incubation for 7 days.The minimum inhibitory concentrations (MICs) were determined after incubation for 7 days at 37°C, defining percent inhibition as 1-(test well fluorescence units/mean FU fluorescence units of triplicate wells containing only bacteria) × 100. The MIC values refer to the lowest concentration at which samples exhibited an inhibition of ≥90% relative to the mean of replicate bacteria-only controls.

Cytotoxicity Assay

Evaluation of the cytotoxic activity of extracts in Vero cells (African green monkey kidney cells) was performed as described previously (Cantrell *et al.*, 1996) using the CellTiter 96 aqueous non-radioactive cell proliferation assay (Promega Corp., Madison, WI). The IC_{50} was defined as the reciprocal dilution resulting in 50% inhibition of the Vero cells. In addition, selectivity index (SI) values were determined as the ratio of cytotoxicity over the MIC. The cytotoxicity was determined by exposing the Vero cells to different concentrations of the extracts. Stock solutions of the extractss were prepared at 10 mg/mL, the positive control Rifampin (RMP) at 50 mg/mL in DMSO. Geometric three-fold dilutions were performed in 96 well clear cell culture plates using the cell culture medium MEM (Gibco, Grand Island, NY) supplemented with 10% of fetal bovine serum (HyClone, Logan, UT). Final DMSO concentrations did not exceed 1% v/v. Extract dilutions were distributed in duplicate in 96-well tissue culture plates (Becton Dickinson Labware, Lincoln Park, NJ) at a volume of 50 µl per well. An equal volume containing 5×10^5 Vero cells (CCL-81; American Type Culture Collection, Rockville, MD) was added to each well and incubated at 37°C in an atmosphere of 5% CO_2 in air. After 72 hours, cell viability was measured using the CellTiter 96 aqueous non-radioactive cell proliferation assay (Promega Corp., Madison, WI) according to the manufacturer's instructions. Absorbance 490 nm was read in a Victor II reader (Perkin Elmer Life Sciences Inc., Boston, MA). The IC_{50} was determined using a Curve-fitting program.

RESULTS

The yield, percentage yield and macroscopic characteristic of extracts of *Uvaria afzelli* root bark and *Tetracera alnifolia* are presented in Table 1. Phytochemical screening of these plants revealed the presence of tannins, triterpenoid saponins, cardiac glycoside and alkaloids. The percentage inhibition of *Mtb* H37Rv ATCC 27294 by the extracts is shown in Table 2.

Table 1
Extractions yield and macroscopic characteristics of crude extracts of *Uvaria afzelii and Tetracera alnifolia*

Sample/solvent	Yield (g)	% Yield	Macroscopic characteristics
Uvaria afzelli root bark			
Chloroform	20.90	5.23	Dark brown shiny congealed mass
Tetracera alnifolia leaf			
n-Hexane	3.58	1.19	Dark green shiny congealed extract
Chloroform	0.53	0.18	Dark green shiny congealed extract
Tetracera alnifolia root bark			
n-Hexane	0.52	0.12	Dark green shiny congealed mass
Chloroform	7.56	1.80	Greenish-brown flakes

The chloroform extract of *Uvaria afzelli*; the hexane and chloroform extracts of *Tetracera alnifolia* root bark had good anti-TB activity with MIC values < 100µg/mL. The leaf extracts of *Tetracera alnifolia* had weak anti-TB activity (MIC >100µg/mL) (Table 3). The Cytotoxicity assay gave IC_{50} values lower than the MIC thus selectivity index (SI= IC_{50}/MIC) was lower than 1(Table 3). Thus the extracts are toxic to the VERO cells and have moderate selectivity for anti-TB activity.

Table 2:
Percentage inhibition of MTB H37Rv by the crude extracts of *Uvaria afzelli* and *Tetracera alnifolia*

Conc. (µg/ml)	100	50	25	12.5	6.25	3.125	1.5625	0.78125	0.390625	
cUa	98	65	15	13	6	4	3	1	1	
hTaL	26	9	6	2	0	0	0	0	0	
cTaL	70	37	34	16	15	9	4	0	0	
hTaR	96	55	52	18	9	5	5	0	0	
cTaR	93	54	53	24	14	12	6	4	3	
Controls(µg/ml)	8	4	2	1	0.5	0.25	0.125	0.0625	0.03125	0.015625
RMP	NT	100	100	100	100	100	98	87	73	48
INH	99	99	98	98	98	98	97	23	3	NT

NOTE: h- hexane, c- chloroform, Ua- Uvaria afzelli, TaL-*Tetracera alnifolia* leaf, TaR-*Tetracera alnifolia* root bark, RMP- rifampicin, INH-isoniazid, NT- Not Tested

Table 3:
Anti-TB activity of extracts of *Uvaria afzelli* and *Tetracera alnifolia* on MTB H37Rv

Plant	Part	Extract	MIC (µg/mL)	IC_{50} (µg/mL)	SI[a]
Uvaria afzelli	root bark	chloroform	87.5	51.69	0.59
Tetracera alnifolia	Leaf	Hexane	>100	n/d	n/d
		chloroform	>100	n/d	n/d
	root bark	Hexane	93.3	14.92	0.16
		chloroform	96.5	9.19	0.09
*RMP			0.07	147.58	2108.29
*INH			0.12	n/d	n/d

*Controls: RMP - Rifampicin, INH - Isoniazid, [a] Selectivity index (SI = IC_{50}/MIC), n/d- not determined

DISCUSSION

The primary treatment for *M. tuberculosis* disease is specific chemotherapy. When antituberculosis drugs are used singly, resistant tubercle bacilli emerge rapidly and multiply. Therefore, drugs are used in combination to yield cure rates of > 95%. However, these regimens are complicated lasting for several months and associated with various adverse effects thus patients compliance to therapy is poor (Fair *et al.*, 2007). This result in the organisms rapidly developing resistance to the chemotherapeutic agents (drugs) used for the treatment of the disease.

The use of herbs for the treatment of tuberculosis is on the increase due to increased incidence of resistance to the available antibiotics. Natural products continue to play a most significant role in the drug discovery and development of highly active antimycobacterial metabolites (Newman and Cragg, 2007; Gibbons, 2005; Pauli *et al*., 2005).

In Nigeria, many medicinal plants have found use in the treatment of microbial infections including tuberculosis. However, there are very few reports on the phytochemical and antimicrobial investigations on these plants. It is our interest to report the anti-tuberculosis activity of these plants since some species in the genera had been reported to possess antimicrobial activity (Mbatchi *et al.*, 2006; Okpekon *et al.*, 2004). Species in the *Uvaria* genus are used in the treatment of wounds, ulcer, inflammation of the face, hands and feet, small pox, leucorrhoea, gonorrhoea, infection of the kidney, bladders and liver, malaria, fever and cough (Kerharo and Borquet, 1950; Oliver, 1960; Borquet and Debray, 1974; Verger, 1995; Kayode *et al.*, 2009). Extracts of *Tetracera* species are used in the treatment of dysentery, urethral discharge, leprosy, toothache and cough (Kerharo and Borquet, 1950; Oliver, 1960; Walker and Sillans, 1960; Kerharo and Adam, 1962).

The activity demonstrated by the extracts is attributable to the presence of tannins, alkaloids and triterpenoid saponins which are secondary metabolites known for their broad-spectrum antimicrobial activity. Asres *et al.,* (2003) reported the potent activity of

tannins isolated from *Combretum molle* against *Mycobacterium tuberculosis*. Alkaloids isolated from *Alstonia scholaris* had activity against *Mycobacterium tuberculosis* as reported by Macabeo *et al.*, (2005) while Wordemichael and colleagues in 2004 reported the activity of triterpenes isolated from *Sapium haematospermum* against *Mycobacterium tuberculosis.*

The present work in accordance with the local uses of these plants demonstrates that *Uvaria afzelli* and *Tetracera alnifolia* may be of therapeutic importance in the treatment of tuberculosis. Further investigations will focus on isolating and identifying the compounds eliciting the activity observed.

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REFERENCES

Akendengue, B., Nguo-Milama, E., Bourobou-Bourobou, H., Essouma, J., Roblot, F., Gleye, C., Laurens, A., Hocquemiller, R., Loiseau, P. and Bories C (2003): Acaricidal Activity of *Uvaria vesicolor* and *Uvaria klaineana* (Annonaceae). *Phytother. Res.* 17: 364-367.

Asres, K., Bucar, F., Edelsbrunner, S., Kartnig, T., Hoger, G. and Thiel W (2001): Investigations of Antimycobacterial Activity of Some Ethiopian Medicinal Plants. *Phytother. Res.* 15:323-326.

Borquet, A. and Debray, M. (1974): In Medicinal Plants of the Ivory Coast. *Trav Doc Orstom* (Serv cent document orstom bondy 93140) 32:1-10.

Cantrell, C.L.; Lu, T., Fronczek, F.R., Fischer, N.H., Adams, L.B. and Franzblau, S.G. (1996). Antimycobacterial cycloartanes from *Borrichia frutescens*. *J Nat Prod.* **59**:1131–1136.

Collins, L. and Franzblau, S. G. (1997): Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and Mycobacterium avium. *Antimicr. Agents Chemother.* 41:1004–1009.

Deng, S., Wang, Y., Inui, T., Chen, S. N., Farnsworth, N. R., Cho, S., Franzblau, S. G. and Pauli, G. F. (2008): Anti-TB Polyynes from the roots of *Angelica sinensis*. *Phytother Res* 22 (7): 878–882.

Fair, E., Hopewell, P. C. and Pai, M. (2007). International Standards for Tuberculosis Care: revisiting the cornerstones of tuberculosis care and control. *Expert Rev. Anti Infect. Ther.* 5:61-5.

Gibbons, S. (2005): Plants as a source of bacterial resistance modulators and anti-infective agents. *Phytochemistry Reviews* 4: 63–78.

Irvine, F. R. (1961): Woody Plants of Ghana. With special reference to their uses. London: Oxford.

Kayode, J., Ige, O. E., Adetogo, T. A. and Igbakin, A. P. (2009): Conservation and Biodiversity Erosion in Ondo State, Nigeria: (3). Survey of Plant Barks Used in Native Pharmaceutical Extraction in Akoko Region. *Ethnobotanical Leaflets* 13: 665-67.

Kerharo, J. and Bouquet, A. (1950): Plantes médicinales et toxiques de la Côte d'Ivoire - Haute-Volta. Vigot Frères, Paris, France; pp 168-240.

Kerharo, J. and Adam, J. G (1962): Premier inventaire des plants medicinnles et toxiques de la Casamance (Senegal). Annales Pharmaceutiques Francaises 20:76-841.

Kihampa, C., Joseph, C. C., Nkunyan, M. H. H., Magesa, S. M., Hassanali, A., Heydenreich, M. and Kleinpeter, E. (2009): Larvicidal and IGR activity of extracts of Tanzanian plants against malaria vector mosquitoes. *J Vector Borne Dis* 46:145-152.

Macabeo, A. P. G., Krohn, K., Gehle, D., Read, R. W., Brophy, J. J., Cordell, G. A., Franzblau, S.G. and Aguinaldo AM (2005): Indole alkaloids from the leaves of Philippine *Alstonia scholaris*. *Phytochemistry* 66 (10):1158-1162.

Mbatchi, S. F., Mbatchi, B., Banzouzi, J. T., Bansimba, T., Nsonde Ntandou, G. F., Ouamba, J-M, Berry, A. and Benoit-Vical, F. (2006): In vitro antiplasmodial activity of 18 plants used in Congo Brazaville traditional medicine. *J. Ethnopharmacol.* 104:168-174.

Newman, D. J. and Cragg, G. M. (2007): Natural products as sources of new drugs over the last 25 years. *J. Nat. Prod.* 70:461–477.

Okpekon, T., Yolou, S., Gleye, C., Roblot, F., Loiseau, P., Bories, C., Grellier, P., Frappier, F., Laurens, A. and Hocquemiller, R. (2004): Antiparasitic activities of medicinal plants used in Ivory Coast. *J. Ethnopharmacol*. 90 (1): 91-7.

Oliver, B. (1960). Medicinal Plants in Nigeria. Nigerian College of Arts, Science and Technology. pp 1-138.

Pauli, G. F., Case, R. J., Inui, T., Wang, Y., Cho, S., Fischer, N. H. and Franzblau, S.G. (2005): New perspectives on natural products in TB drug research. *Life Sciences* 78:485–494.

Trease, TTrease, E. and Evans, W. C. (1989): Pharmacognosy. 13th edition. Bailliere-Tindall. pp 388, 396, 502-546.

Verger, P. F. (1995): Ewé: The use of plants in Yoruba society. Editoria Schwarcz, Sao Paulo, pp 744.

Walker, A. R. and Silans, R. (1961): Les plantes utiles du Gabons. Paul Lechevalier, Paris. pp 19-132.

Woldemichael, G. M., Gutierrez-Lugo, M. T., Franzblau, S. G., Wang, Y., Suarez, E. and Timmermann, B. N. (2004): *Mycobacterium tuberculosis* Growth Inhibition by Constituents of *Sapium haematospermum. J. Nat. Prod.* 67 (4):598–603.

World Health Organization (WHO) (2009): Global tuberculosis control: epidemiology, strategy, financing, pp. 6–33.

World Health Organization (WHO) (2007): Fact Sheet N° 104, pp1-2.