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Full Length Research Paper

In vitro antimycobacterial studies on the leaf extracts and fractions of *Pavetta crassipes* K. Schum

Nneka N. Ibekwe[1,2]*, Abayomi T. Orishadipe[1], Helena Boshoff[3], Akinbo A. Adesomoju[2], Joseph I. Okogun[1] and Clifton E. Barry[3]

[1]National Institute for Pharmaceutical Research and Development, Garki, Abuja, Nigeria.
[2]Department of Chemistry, University of Ibadan, Oyo State, Nigeria.
[3]Tuberculosis Research Section, Laboratory of Clinical Infectious Diseases, National Institute for Allergy and Infectious Diseases, NIH, Bethesda, MD, USA.

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Tuberculosis is a major public health threat and Nigeria has the world's fifth largest tuberculosis burden, with nearly 311,000 estimated cases annually. *Mycobacterium tuberculosis*, the causative organism, has acquired resistance against most of the current chemotherapy, thereby calling for the urgent development of new antituberculosis drugs. Preliminary phytochemical and antimycobacterial studies were carried out on *Pavetta crassipes*, an ethnomedicinal plant, used in Nigeria by Traditional Medicine Practitioners (TMPs) to treat tuberculosis. The leaf extracts showed *in vitro* antimycobacterial activity with the methanol and ethyl acetate extracts being most active with MICs of 250 and 521 µg/ml, respectively. Further fractionation of the methanol and ethyl acetate extracts yielded five fractions with MICs ≤ 900 µg/ml. One of the ethyl acetate fractions had the most potent activity with MIC of 200 µg/ml.

Key words: *Mycobacterium tuberculosis*, *Pavetta crassipes*, antimycobacterial activity, bacilli galmette guerin (BCG).

INTRODUCTION

Tuberculosis (TB), a contagious, infectious disease mainly caused by *Mycobacterium tuberculosis*, is a public health threat in both developing and developed nations. Nigeria has the world's fifth largest tuberculosis burden, with nearly 311,000 estimated cases annually (WHO, 2008). Effective treatment of TB has been hampered by the emergence of drug resistant strains of *M. tuberculosis*, calling for the search for new drugs. Plants are a proven source of novel and effective drugs as exemplified by artemisinin and taxol. A number of plants with promising activity against *M. tuberculosis* have been documented (Copp and Pearce, 2007; McGaw et al., 2008).

*Corresponding author. E-mail: nnekaibekwe@yahoo.com. Tel: +234-805-893-988.

Pavetta crassipes K. Schum (member of the family Rubiacaea) is a plant claimed as a traditional cure for TB and other respiratory diseases in Northern Nigeria. It is a glabrous shrub that grows to 6 m high, trunk to 30 cm girth, of the savanna (Burkill, 1997). The leaf is routinely used in Nigeria for the management of respiratory disorders and hypertension in ethnobotanical practice. *P. crassipes* leaves, locally known as Gadu in Northern Nigeria, are typically eaten as food or used for the treatment of fever, schistosomisasis, mental illness, convulsions, malaria, hookworms (Amos et al., 1998) and pains (Abubakar et al., 2007). The following pharmacological activities have been reported on the plant; anti-plasmodial/ malarial activity (Sanon et al., 2003), hypotensive activity (Amos et al., 2003), inhibitory effects on gastrointestinal and uterine smooth muscles (Amos et al., 1998) and *in vitro* antiprotozoal, antimicrobial and antitumor activities (Balde et al., 2010).

However, the antimycobacterial activities of *P. crassipes* have not been previously reported. Phytochemical screening and antimycobacterial studies of the extracts are described and reported herein.

MATERIALS AND METHODS

Analytical grades of organic solvents; hexane, ethyl acetate and methanol (Sigma-Aldrich) were used for extraction. Silica gel F_{254} (0.25 mm) prepared for TLC and silica gel 60 (70 to 230 mesh) for column chromatography were purchased from Merck. Spots were visualized by spraying with vanillin-sulphuric acid, followed by charring at 100°C for 10 min. Reagents used for phytochemical screening of extracts were freshly prepared using standard methods.

Plant material

The fresh leaves of *P. crassipes* were collected in Abuja in October, 2007 and the species was identified by Jemilat Ibrahim, a taxonomist at the National Institute for Pharmaceutical Research and Development, Abuja, Nigeria. A labeled voucher specimen, NIPRD/H/6241, was deposited in the Institute's Herbarium.

Extraction

Aqueous methanol extraction

Air-dried and powdered leaves (10 g) of *P. crassipes* were covered in 100 ml of 70% aqueous methanol, and left overnight. The extract was concentrated at 40°C under reduced pressure to remove the methanol, and then lyophilized, to yield dry powder.

Successive organic extraction

The air-dried and powdered leaves (1 kg) of *P. crassipes* were extracted sequentially by cold maceration with hexane, ethyl acetate and methanol between intervals of 24 h. The extracts were concentrated at 40°C under reduced pressure to yield the hexane, ethyl acetate and methanol extracts.

Phytochemical screening of extract

Preliminary phytochemical investigations were performed on the aqueous methanol extract, using standard methods (Agraval and Paridhavi, 2007).

Fractionation of methyl and ethyl acetate extracts of *P. crassipes*

The ethyl acetate extract (9 g) was fractionated using silica gel column chromatography, with gradient elution of n-hexane, EtOAc and MeOH (hexane-EtOAc, 90:10 to hexane-EtOAc, 0:100 to EtOAc-MeOH, 90:10 in 10% increments of the more polar solvent) to obtain 14 major fractions, PCE_1 to PCE_{14} based on similarities in TLC profiles.

The methanol extract (10 g) was fractionated using silica gel column chromatography, with a gradient elution of n-hexane, EtOAc, and MeOH (hexane-EtOAc, 90:10 to hexane-EtOAc, 0:100 to EtOAc-MeOH, 50:50 in 10 to 20% increments of the more polar solvent). Thirteen major fractions, PCM_1 to PCM_{13} were collected based on similarities of their TLC profiles.

Determination of minimum inhibitory concentration (MIC)

A modified broth microdilution method was employed (Coban et al., 1993) for preliminary screeening, and bacilli galmette guerin (BCG) and *M. tuberculosis* H37Rv were the strains used. The organism was subcultured into Middlebrook 7H9 broth in screw capped tubes and incubated at 37°C for 7 days. Growth was observed and the tubes were then left at room temperature for the solid particles to sediment. The homogenous suspension at the upper part of the tube was then transferred into sterile screw capped tubes and measured at an optical density of 0.2 to 0.3 at 650 nm after which cells were diluted 1000-fold in 7H9-medium.

Susceptibility testing was performed in clear 96-well plates containing 50 µl of 7H9 liquid medium in each well except the first well. Extracts and fractions for the antimycobacterial assay were prepared at varying concentrations in 100% DMSO. The plant extract (fraction)-medium) was vortexed and 100 µl of it dispensed into the first column (of the first row). 50 µl of extract solution was then transferred from column 1 into column 2, and similarly until the last column to give two-fold serial dilutions.

The diluted inocula (50 µl) were dispensed into each of the wells, giving a final volume of 100 µl per well. This caused a further 2-fold dilution of the potential inhibitors in the wells. The plates covered in Ziploc bags, were incubated for 5 to 7 days at 37°C. Isoniazid was used as positive control while the cells were used as negative control. The assay was carried out in duplicate. MIC values were determined as the lowest concentration that inhibited the complete visible growth of the organism.

RESULTS

Ten grams of air-dried ground leaves of *P. crassipes* on aqueous methanol extraction, yielded 1.31 g of extract. One kilogram of air-dried ground leaves of *P. crassipes* yielded 10.4 g of hexane extract, 16.8 g of ethyl acetate extract and 131.7 g of methanol extract, representing an extraction yield of 1.04, 1.68 and 13.2% respectively.

Results of the phytochemical screening of the aqueous methanol extract of *P. crassipes* revealed the presence of tannins, saponins, alkaloids, flavonoids, reducing sugars, anthraquinones and terpenes/ steroids (Table 1).

Antimycobacterial studies carried out on the extracts employing the broth microdilution method (BMM) showed that the aqueous methanol extract had weak activity against BCG with an MIC of 6.5 mg/ml. The organic methanol and ethyl acetate extracts were more active against BCG with MICs of 521 and 250 µg/ml, respectively.

Bio-assay of fractions of the active extracts against *M. tuberculosis* H_{37}Rv, as shown in Table 2, revealed that for the ethyl acetate extract, the best antimycobacterial activity at 500 µg/ml, was eluted with 10% methanol in ethyl

Table 1. Phytochemical screening for metabolites of *P. crassipes.*

Test	Methanol extract
Tannins	+
Saponins	+
Alkaloids	+
Flavonoids	+
Carbohydrates	+
Terpenes/ Steroids	+
Anthraquinones	+
Cardiac glycosides	+

Key: + = positive.

Table 2. Antimycobacterial activities of active fractions against *M. tuberculosis* $H_{37}Rv$.

Extract	Fractions	MIC (µg/ ml)	Mobile phase
EtOAc	14	500	10% MeOH in EtOAc
CH_3OH	1	250	5 - 10% EtOAc in Hex
CH_3OH	3	325	10% EtOAc in Hex
CH_3OH	4	200	30% EtOAc in Hex
CH_3OH	8	900	100% EtOAc

acetate. In the methanol extract, the activities were distributed across the various fractions eluted between 5% ethyl acetate in hexane to 100% ethyl acetate with MICs ≤ 900 µg/ml. The fraction eluted with 30% ethyl acetate in hexane was however the most active with an MIC of 200 µg/ml.

DISCUSSION

The phytochemical screening carried out on the aqueous methanol extract of *P. crassipes* leaves showed that it contained a cocktail of the major classes of natural products. The antimycobacterial assays also showed that the organic extracts had more potent activity than the aqueous alcoholic extract. The antimycobacterial activities observed in the ethyl acetate and methanol extracts indicated that more than one class of compounds may be responsible for the observed activities. The biological activity observed in several fractions of the methanol extract may be as a result of synergism by several metabolites, or as a result of a single major component of the extract cutting across the different fractions. Studies are ongoing in our laboratory to isolate and characterize the bioactive compounds. Some excellent review articles have been published on some classes of compounds such as terpenoids, alkaloids, coumarins/chromones, phenolics and straight chain hydrocarbons, with antimycobacterial activities (Okunade et al., 2004; Copp, 2003; Copp and Pearce, 2007).

Conclusion

Extracts and fractions of the leaves of *P. crassipes* leaves showed potent antimycobacterial activities. Results have shown that strong positive correlation exists between ethnomedicinal claims of this plant as an antituberculosis agent, and *in vitro* antimycobacterial activities.

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