

ANTIMYCOBACTERIAL AND CYTOTOXICITY EVALUATION OF THE CONSTITUENTS OF *MONODORA CAROLINAE*

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ABSTRACT

Phytochemical investigation of the stem bark of the recently described species Monodora carolinae (Annonaceae) afforded prenylindole derivatives (E)-4-(1H-indol-6-yl)-but-3-en-2-one (1), 5-formylindole (2), fatty acid (Z)-hexade-7-enoic acid (3) and the lignanamide cannabisin B (4). The structures of the isolated compounds were established using NMR and MS analyses. The antimycobacterial activities of the extracts and isolated compounds were evaluated against Mycobacterium madagascariense (MM) and M. indicus pranii (MIP) using the two-fold microtitre dilution method. While the extracts exhibited minimum inhibitory concentration (MIC) ranging from 500 – 1000 µg/mL, the isolated compounds were 125 – 250 µg/mL, indicating very low activity. Cytotoxic effects were evaluated using brine shrimp larvae whereby the ethanolic extract of the root bark exhibited the lowest LC₅₀ (< 3µg/mL). Isolation of prenylindole derivatives is of chemotaxonomic significance that affirms taxonomic placement of this plant species into the genus Monodora. This is the first time cannabisin B is reported from the genus Monodora.

Keywords: *Monodora carolinae*, Annonaceae; prenylindole, cannabisin B, (Z)-hexade-7-enoic acid; antimycobacterial, cytotoxicity.

INTRODUCTION

Tuberculosis (TB), the deadly infectious disease caused by bacillus *Mycobacterium tuberculosis*, is the second leading cause of death worldwide from a single human pathogen following HIV (WHO 2011). In 2010, 8.8 million TB incidents were reported worldwide, with approximately 3 million being lethal (WHO 2011). In Africa the number of deaths caused by TB is 254,000 per annum of which an estimated 32,000 occur in Tanzania alone, of which most are individuals co-infected with HIV (WHO 2009). The emergence of new resistant strains such as the multidrug resistant (MDR) and extremely multidrug resistant (XMDR) coupled with the high toxicity of TB drugs have accelerated the

need for alternative agents (WHO 2010, Higuchi *et al.* 2009). Given the tremendous chemical diversity of plants, the World Health Organisation (WHO) has recommended exploration of herbs or plants when searching for efficient chemotherapies for infectious diseases.

Previous studies on annonaceous plant species found among others in Tanzania (Nkunya 2005) yielded nitrogenous compounds possessing antimycobacterial properties, such as cleistopholine isolated from *Cleistopholis patens* (Waterman and Muhammad 1985), sampagine from *Cananga odorata* (Rao *et al.* 1986) and bidebiline E from *Polyalthia cerasoides* (Kanokmedhakul *et al.* 2007). Species of the genus *Monodora* have also been reported to accumulate alkaloids, the major constituents being prenylated indoles, which are considered as chemotaxonomic markers of

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the genus (Nkunya *et al.* 2004; Nkunya 2005). The tendency of the members of the genus *Monodora* to accumulate alkaloids with some exhibiting antimycobacterial activities prompted us to investigate the recently described plant species *M. carolinae* for antimycobacterial and cytotoxic properties, and to further elaborate on the understanding of the chemotaxonomy of the genus *Monodora*. *M. carolinae* is a near-endemic plant species in Tanzania. It is found on Matumbi Hills in Kiwengoma Forest, Rufiji District, Pwani Region as well as the Rondo Plateau in Southern Tanzania and Northern Mozambique (Couvreur *et al.* 2006). This small tree or large shrub grows in coastal thickets on deep leached sandy soils and has been categorized as an endangered plant due to its decreasing population (Couvreur *et al.* 2006; Eastern Arc Mountains & Coastal Forests CEPF Plant Assessment Project 2009). No ethnomedical use of the plant species has been reported so far, however, the stem is used by villagers for making cooking spoon. This paper hereby reports for the first time the phytochemical investigation and the evaluation of antimycobacterial and cytotoxic activities of the constituents from this plant species.

MATERIALS AND METHODS

General Information

Column chromatography: Silica gel 60 (Merck 230-400 mesh) and Sephadex[®] LH-20 (Pharmacia); TLC: Pre-coated plates (Merck, Kieselgel 60 F₂₅₄, 0.20 mm); Visualization: UV (254 and 365 nm) followed by anisaldehyde spray reagent/heat at *ca.* 110 °C; IR (ν_{max} , cm⁻¹): Bruker FT Spectrophotometer; ¹H and ¹³C-NMR: 300 MHz Bruker and 600 MHz Varian spectrometers; CDCl₃ and CD₃OD were used as solvents and TMS as an internal standard; MS: Recorded at 5.03 Kv ESI 93.8 TOF mass spectrometer. Extraction and chromatographic solvents and glycerol (AR) were purchased from Lab equip Ltd

(Tanzania), Middlebrook 7H9 broth base was obtained from HIMEDIA India, iodinitrotetrazolium (INT) chloride and Ciprofloxacin (R&D) were purchased from Sigma (UK). The 96 wells microtiter plates were supplied by KAS Medics (Tanzania).

Plants Materials

The stem, roots and leaves of *M. carolinae* plant species were collected in November 2011 from Matumbi Hill in Kiwengoma Forest, Rufiji District, Pwani Region, Tanzania. The plants species was identified and authenticated by Mr. F.M. Mbago, a taxonomist at the Herbarium of the Botany Department, University of Dar es Salaam where a voucher specimen (FMM 3582) was deposited.

Extraction and Purification

The air dried pulverized leaves, stem and root barks of *M. carolinae* were sequentially extracted in petroleum ether, dichloromethane and ethanol at a room temperature for 48 h consecutively. The extracts were then concentrated using rotary evaporator at a reduced pressure to afford the crude extracts, which were kept refrigerated until required for further investigation. The dichloromethane extract (22g) was adsorbed on silica gel and subjected to silica gel gravitational column chromatography eluted by 5 - 25% polarity gradient mixtures of ethyl acetate/CH₂Cl₂ giving 40 fractions, which were then combined into four fractions based on similarities observed under thin layer chromatography (TLC) analysis. The 2nd fraction was subjected to Sephadex LH-20 gel filtration eluted with 1:1 methanol/CH₂Cl₂ affording (*E*)-4-(1*H*-Indol-6-yl)-but-3-en-2-one [**1**, UV absorbing (254 nm) and fluorescing blue (365 nm); orange when sprayed with *p*-anisaldehyde reagent + heating] as a major product together with (*Z*)-hexadec-7-enoic acid (**3**, brown when sprayed with *p*-anisaldehyde reagent + heating). The 4th fraction was also subjected

to gravitational column chromatography in silica gel and eluted using 5 - 25% polarity gradient ethyl acetate/CH₂Cl₂ followed by further purification on Sephadex LH-20 eluted with MeOH yielding 5-formylindole (**2**, UV absorbing (254 nm) and fluorescing blue (365 nm); orange when sprayed with *p*-anisaldehyde reagent + heating). The ethanolic extract (25 g) was subjected to a repeated silica gel gravitational column chromatography and eluted using an increasing polarity of solvent systems ranging from 30 - 60% ethyl acetate/petroleum ether. The obtained fractions were then combined upon TLC analysis and further purified on Sephadex[®] LH-20 yielding cannabisin B [**4**, UV absorbing (254 nm) and fluorescence (365 nm); orange when sprayed with *p*-anisaldehyde reagent + heating]. **(E)-4-(1H-Indol-6-yl)-but-3-en-2-one (1)**. Yellow solid; yield: 31 mg; ¹H and ¹³C NMR (CD₃Cl) data in good agreement with literature (Nkunya *et al.* 2004). **5-Formylindole (2)** Yellow solid, yield 22 mg; ¹H and ¹³C NMR (CD₂Cl₂) data in good agreement with literature (Nkunya *et al.* 2004). **(Z)-Hexadec-7-enoic acid (3)** Colorless oil; yield 9.2 mg; ¹H and ¹³C NMR (CD₂Cl₂) data consistent with the structure for compound **3** (Domergue *et al.* 2003). **Cannabisin B (4)** White amorphous solids, yield 40 mg; IR ν_{\max} 3377.50 (OH, NH), 1726.98, 1660 (C=O), 1615.33, 1515.33 (olefin/benzene ring); HRESIMS *m/z* 599.2792 [M+3H]⁺, for molecular formula C₃₄H₃₃N₂O₈; ¹H and ¹³C NMR (CD₃OD) data in good agreement with literature Sakakibara *et al.* 1992).

Antimycobacterial assay

The antimycobacterial assay was carried out at Institute of Traditional Medicine (ITM), Muhimbili University of Health and Allied Sciences (MUHAS), Tanzania. The antimycobacterial efficacy of the crude extracts and pure isolated compounds from *M. carolinae* were obtained using two non-

pathogenic mycobacteria namely *Mycobacterium madagascariense* (DSM 44641) and *M. indicus pranii* (DSM 45239) supplied by DSMZ – The Germany Resource Centre for Biological Material, Braunschweig, Germany. The mycobacterium strains were sub-cultured in Middlebrook 7H9 broth base supplemented with glycerol. 1.18 g of Middlebrook 7H9 broth base was suspended in 230 ml of distilled water in a Scotch bottle (500 ml) followed by addition of 1 ml of glycerol. The mixture was heated to dissolve the broth base completely, thereafter autoclaved at 121 °C for 15 minutes. The mixture was left to cool to 31 and 35 °C under laminar flow, before separately being inoculated with *Mycobacterium madagascariense* (MM) and *Mycobacterium indicus pranii* (MIP), respectively. Thereafter, MM was incubated at 31 °C and MIP at 37 °C. The optimal growths of the bacteria cultures were observed after 5 days and thus were ready for anti-mycobacterial assays. The Minimum Inhibitory Concentration (MIC) values of crude extracts and pure compounds against the two *Mycobacterium* strains were determined by two fold microtitre dilution method (Eloff 1998).

Brine Shrimp Lethality Test (BSLT):

The BSLT was performed at the Chemistry Department, University of Dar es Salaam (UDSM) following standard procedures (Meyer *et al.* 1982). The brine shrimp (*Artemia salina*) Leach larvae indicator animals were used for preliminary cytotoxicity assay of the crude extracts. The stock solutions of all extracts (20 mg/mL) were prepared in DMSO. Crude extracts were tested at concentrations of 500, 50 and 5 mg/mL in triplicate vials containing 10 brine shrimp larvae hatched under light for 24 hours. The negative control contained brine shrimp, artificial sea water and 1% DMSO. Using KaleidaGraph 4.5 computer programme, the LC₅₀ values were eventually deduced from the regression equation

obtained by plotting percentage mortality against logarithm of the concentration of the sample tested.

RESULTS AND DISCUSSION

The results for cytotoxicity of *Monodora carolinae* leaf, stem and root bark crude extracts are presented in Table 1. The

ethanolic extract of root bark was most cytotoxic with $LC_{50} < 3 \mu\text{g/ml}$. The other crude extracts exhibited toxicity in the 29-189 $\mu\text{g/ml}$ LC_{50} range, with the petroleum ether stem extract being the least active. Higher toxicity exhibited by ethanolic root extract as compared to the rest, could be associated with its polar constituents.

Table 1: Cytotoxic Activity of Crude Extracts from *M. carolinae*

Extract	MCSP	MCSD	MCSE	MCLP	MCRD	MCRE	MCLE	MCRP
LC_{50} ($\mu\text{g/mL}$)	NA	47	144	189	181	< 3	44	102
R	0.99	0.87	0.92	0.97	0.62	0.95	0.86	0.95

MCSP = Pet ether extract of the stem bark of *M. carolinae*; MCSD = Dichloromethane extract of the stem bark of *M. carolinae*; MCSE = Ethanol stem extract of *M. carolinae*; MCLP = Pet ether leaves extract of *M. carolinae*; MCLE = Ethanol extract of the leaves of *M. carolinae*; MCRP = Pet ether extract of the root bark of *M. carolinae*; MCRE = Ethanolic extract of the root bark of *M. carolinae*. R = Linear regression; NA = Not Active.

The evaluation of antimycobacterial activity of the crude extracts of *M. carolinae* against the fast growing non-pathogenic *Mycobacterium* species, *M. madagascariense* (*MM*) and *M. indicus pranii* (*MIP*) revealed low levels of efficacy as indicated by their Minimum Inhibitory Concentrations (MICs) (Table 2). The ethanolic stem bark extract inhibited growth of both *MM* and *MIP* at MIC values of 500 $\mu\text{g/mL}$ and 1000 $\mu\text{g/mL}$, respectively. The dichloromethane extract of stem bark exhibited selective activity against *MM* with MIC of 500 $\mu\text{g/mL}$ but showed no activity against *MIP*. Dichloromethane root and pet

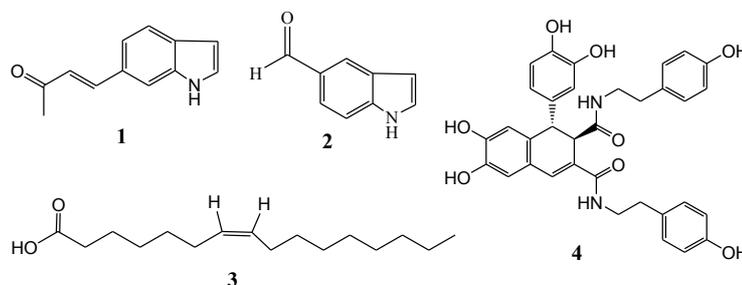
ether leaves extracts also showed low against *MM* at an MIC value of 500 $\mu\text{g/mL}$ each. On the other hand, the petroleum ether extract from stem bark had no activity to both *Mycobacterium* species tested. Despite root bark extracts showing strong cytotoxicity (Tables 1), it was not possible to chemically investigate it further due to paucity of the plant materials. Thus, attention was directed to the stem bark extracts for further chromatographic purification in order to unravel the chemical constituent of the plant species and additionally evaluate their biological properties.

Table 2: Antimycobacterial Activity of the Crude Extracts of *M. carolinae*

Extract		MCSP	MCSD	MCSE	MCLP	MCRD	MCRE	MCLE	Ciprofloxacin
MIC ($\mu\text{g/mL}$)	<i>MM</i>	NA	500	500	500	500	NA	NA	50
	<i>MIP</i>	NA	250	1000	NA	1000	NA	NA	10

NA = Not Active

Repeated column chromatography of the dichloromethane extract of the stem barks of *M. carolinae* yielded two modified prenylated indoles (*E*)-4-(1*H*-indole-6-yl)-but-3-en-2-one (**1**) and 5-formylindole (**2**), both compounds previously being reported from *M. angolensis* (Nkunya *et al.* 2004), and a fatty acid, (*Z*)-hexadec-7-enoic acid (**3**) (Domergue *et al.* 2003). On the other hand, repeated chromatography of the ethanolic extract of the stem bark of *M. carolinae* afforded an arylnaphthalenelignamide cannabisin B (**4**) that has previously only been isolated from



the seeds of *Cannabis sativa* (Cannabidaceae) (Sakakibara *et al.* 1992) and *Xylopia aethiopica* (Annonaceae) (Lajide *et al.* 1995). To the best of our knowledge, this is the first report of the presence of cannabisin B in a member of *Monodora* plant species and second to the family Annonaceae. The identities of the isolated compounds were verified by detailed analysis of their 1D (^1H and ^{13}C NMR) and 2D (COSY, HMQC and HMBC) spectroscopic as well as spectrometric data, which were consistent with those reported in the literatures.

The isolated compounds were then investigated for their anti-mycobacterial activity against *M. madagascariense* and *M. indicus pranii*, the results of which are presented in Table 3. Thus, (*E*)-4-(1*H*-indol-6-yl)-but-3-en-2-one (**1**) exhibited selective activity against tested *Mycobacteria* species, whereby low activity was observed against *M. indicus pranii* at an MIC value of 250 $\mu\text{g/mL}$, but remained essentially inactive against *M. madagascariense*. The alkaloid 5-formylindole (**2**) also exhibited low activity against both *M. madagascariense* and *M. indicus pranii* (MIC value 125 $\mu\text{g/mL}$). The

slightly higher activity of 5-formylindole relative to compound **1** could be attributed to the higher electrophilicity nature of the aromatic aldehyde functionality in **2** compared to the α,β -unsaturated keto functionality in compound **1**. Compounds **1** and **2**, which previously have been reported from *M. angolensis* (Nkunya *et al.* 2004), have never been reported for any biological activity before, hence their antimycobacterial evaluation are hereby reported for the first time.

Table 3: Antimycobacterial Activity of the Pure Compounds from *M. carolinae*

Compound		1	2	3	4	Ciprofloxacin
MIC ($\mu\text{g/mL}$)	MM	NA	125	250	250	50
	MIP	250	125	NT	250	10

NA = Not Active, NT = Not tested

In these antimycobacterial assays, the fatty acid **3** exhibited low activity towards *MM* with an MIC value of 250 mg/mL, but the compound was not tested against *MIP* due to inadequate amount isolated. The unsaturated fatty acids such as **3** are ubiquitous to plant species most of them differing on the degree of unsaturation, chain length and position of unsaturation. Unsaturated fatty acids with 16 - 20 carbons are reported to be highly active against particular species of *Mycobacterium*, good examples, being oleic and palmitoleic acids envisaged to disrupt the bacterial cell membrane resulting in a change in membrane permeability (Carballeira 2008). Therefore, the same mechanism could have also attributed to the observed antimycobacterial activity of compound **3** reported in these investigations, though with very low efficacy. Similarly, cannabisin B (**4**) exhibited low activity against both *M. madagascariense* and *M. indicus pranii* with an MIC value of 250 µg/mL. Previous studies have indicated the compound to exhibit antifeedant (Lajide *et al.* 1995) and antioxidant (Chen *et al.* 2012) properties, and more recently as anticancer agent (Chen *et al.* 2013).

CONCLUSION

The antimycobacterial activities of the tested compounds and extracts were extremely lower than that of the standard drug ciprofloxacin (MIC value of 10 and 50 µg/mL against *MIP* and *MM*, respectively), indicating constituents of *M. carolinae* evaluated in these investigations to be non-potential candidates for anti-tubercular applications. The cytotoxic activity of its extracts suggests the constituents of the plant species could find other applications as anticancer or pesticide agents. Furthermore, isolation of prenylated indole derivatives from *M. carolinae* is of chemotaxonomic significance as it further affirms taxonomic placement of this recently described plant species into the genus *Monodora* whereas isolation of cannabisin B for the first time

from this genus further indicates its chemodiversity. This is the second encounter of lignamide cannabisin B (**4**) from the family Annonaceae, previously being reported from *Xylopia aethiopica* (Annonaceae, sub-family Annonoideae) (Lajide *et al.* 1995) and *Cannabis sativa* (Cannabidaceae) (Sakakibara *et al.* 1992) while similar compounds are reported from other six different plant families (Lajide *et al.* 1995), suggesting chemotaxonomic relationship between the species within the same family and beyond that inquires further chemotaxonomic studies.

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