A triterpene ketone from an anti-mycobacterial tuberculosis chromatography eluate from the n-hexane fraction of the fruits of *Harungana madagascariensis* Lam. Ex Poiret (Hypericaceae)

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Abstract

Clinical cases of drug resistant tuberculosis (TB) are a global threat because of the cost and extended duration of treatment regimen. This underscores the continuous drive to discover new therapeutic agents that are faster, simpler and affordable often by recourse to natural products. This report highlights the potentials of the Nigerian medicinal plant *Harungana madagascariensis* Lam. Ex Poiret (Hypericaceae) as a source of lead agents for the discovery and development of anti-tuberculosis drugs. The fruit of *H. madagascariensis* was extracted with 70% aqueous ethanol by cold maceration. The crude 70 % aqueous ethanol extract was partitioned with n-hexane to give n-hexane (NHF) soluble portions. The NHF was fractionated further on a column packed with normal phase silica gel (200-400 Mesh size) to afford the chromatography fractions used in this study. The structure of isolated compound was elucidated using spectroscopic (NMR, IR, and MS) techniques. Anti-*Mycobacteria tuberculosis* susceptibility screening of the chromatography fractions was done using Lowenstein Jensen method with rifampicin, ethambutol, isoniazid and dihydrostreptomycin as reference anti-TB drugs for comparison. Four fractions NHF1-4 were obtained from the NHF. The chromatography fractions: NHF2 and NHF3 inhibited the growth of the *Mycobacteria tuberculosis*. From the NHF2 was isolated the known bioactive triterpene ketone friedelan-3-one. This preliminary screening underscores the potentials of this ethno medicinal plant as a source of lead compounds for the development of anti-TB drugs.

Keywords: *Harungana madagascariensis*; Hypericaceae; Fruits; Anti-tuberculosis agents; Friedelan-3-one

1. Introduction

Tuberculosis (TB), a chronic and deadly infectious disease caused by the bacillus *Mycobacterium tuberculosis* infects one third of the world population and kills about 15 million people yearly. The global World Health Organization (WHO) reports showed that an estimated 10 million people developed TB with 1.4 million deaths recorded in 2019. Men accounted for 56%, women 32%, and children 12% [1]. Multi-Drug Resistance TB (MDR-TB) is defined by resistance to
the two most commonly used drugs (isoniazid and rifampicin) in the current four drug (or first line) regimen, while a TB strain is called Extensively Drug Resistance -TB (XDR-TB) when it becomes resistant to the four first line drugs. This is a huge global threat because of the cost of the medicine and number of years involved for the drug to work. A 2015 United Kingdom parliamentary group report by Astrid [2] stated that MDR-TB could cost the world $16.7 trillion by 2050. Thus, new treatments that are faster, simpler and affordable are urgently needed.

Nature is blessed with plant secondary metabolites with diverse biological activities including anti-microbial such as anti-Mycobacteria tuberculosis among others. Research into natural products that has antimicrobial activities have contributed immensely to the discovery and development of drugs for the treatment of infections caused by pathogenic microbes. Harungana madagascariensis, a tropical shrub found in Africa has been used in ethnomedicinal practice to treat bacterial infections [3-4], gastrointestinal diseases [5] as well as cough and bronchial distress [6]. This preliminary work reports the anti-Mycobacteria tuberculosis potentials of the fruits of H. madagascariensis.

2. Material and methods

2.1. Materials

Reagents and solvents used in this study were of analytical grade and are products of JHD Chemicals, China. Standard control drugs used include ethambutol [99%], rifampicin [99%], isoniazid [99%] and dihydrostreptomycin [99%]. The clinical strain of Mycobacteria tuberculosis used were supplied and cultured by the Tuberculosis Reference Laboratory, Zankli Research Centre, Bingham University, Nassarawa State, Nigeria.

2.2. Sample collection and extraction

The matured fruits of H. madagascariensis were collected from the Medicinal plant Garden of the Department of Pharmacognosy and Phytotherapy, University of Port Harcourt, Nigeria and identified by Taxonomist with voucher specimen deposited at the herbarium of the same department. It was air dried and pulverized. A 600 g quantity of the pulverized sample was extracted with 70% aqueous ethanol by cold maceration for 72 hours with filtration and change of solvent done every 24 hours. After the extraction, it was concentrated with rotary evaporator at 40°C to at least one-tenth of its volume. The crude aqueous ethanol extract was partitioned exhaustively with n-hexane to obtain the lipid-rich n-hexane fraction (NHF) used for this study.

2.3. Fractionation of N-hexane fraction

Separation of NHF was done using chromatography techniques. Briefly, 6.2 g of NHF was pre-adsorbed by mixing in silica gel (4 g, normal phase, Mesh 200-400) and loaded on a column (internal diameter 4 cm), dry packed with same silica gel as adsorbent to a height of 15 cm. The mobile phase gradient (500 ml of each) used comprised of n-hexane (4:0 v/v); n-hexane: DCM (3:1, 2:2, 1:3, 0:4 v/v); DCM: ethanol (3:1, 2:2 v/v). The eluted fractions were collected at 10 ml intervals and pooled based on observed Rf of resolved spots and color reaction with chromogenic spray reagent from TLC. Four pooled fractions were obtained and were designated NHF 1-4. All the fractions were screened for anti-Mycobacteria tuberculosis activity.

2.4. Anti-Mycobacteria tuberculosis susceptibility test

This was done using the Lowenstein-Jensen solid based agar dilution method reported by Selvakumar [7]. Briefly, the freshly prepared LJ medium (100 ml) was mixed with the test (chromatography fraction) sample (1 mg/ml, 1 ml). About 7-8 ml of the reconstituted test sample-LJ mixture was then dispensed into sterile bijou tubes and allowed to inspissate for 45 minutes on a water bath at 85°C in a slanting position. The reference standard drugs: Isoniazid (0.02 mg/ml), dihydrostreptomycin (0.80 mg/ml), ethambutol (0.20 mg/ml) and rifampicin (8.00 mg/ml) were similarly reconstituted as described for the test samples. 1.0 McFarland of de-contaminated clinical isolates of Mycobacterium tuberculosis (MTB) was prepared in sterile distilled water while 10 µL of the inoculum concentrations was inoculated on the separate standard drugs, phytodrugs, and negative control reconstituted LJ media slant and incubated for six weeks at 37°C. Each sample analysis was done in duplicate. Where a test substance showed inhibition with greater than 19’colonies, it is taken as not being active. Where no colony growth was observed, the test substance is taken as bactericidal.
3. Results

3.1. Bioactivity of fractions from n-Hexane extract

Table 1 Anti-\textit{Mycobacteria tuberculosis} activities of chromatography fractions from n-hexane soluble portion (NHF) of \textit{H. madagascariensis} fruits

<table>
<thead>
<tr>
<th>Test samples</th>
<th>Eluting mobile phase gradient</th>
<th>Anti-\textit{Mycobacteria tuberculosis} activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHF1</td>
<td>n-hexane: Dichloromethane (4:0 v/v)</td>
<td>Inc</td>
</tr>
<tr>
<td>NHF2</td>
<td>n-hexane: Dichloromethane (3:1-1:3 v/v)</td>
<td>+</td>
</tr>
<tr>
<td>NHF3</td>
<td>n-hexane: Dichloromethane (0:4 v/v)</td>
<td>+</td>
</tr>
<tr>
<td>NHF4</td>
<td>Dichloromethane: Ethanol (3:1-2:2 v/v)</td>
<td>-</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>Not applicable</td>
<td>+</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>Not applicable</td>
<td>+</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>Not applicable</td>
<td>+</td>
</tr>
<tr>
<td>Dihydrostreptomycin</td>
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</tbody>
</table>

NHF1-4 are chromatography fractions from NHF; Inc = inconclusive; + = active; - = inactive

3.2. Characterization of isolated compound, friedelan-3-one

Table 2 NMR spectra data of the compound isolated from NHF2 of \textit{H. madagascariensis} fruits compared with that of friedelin in Literature

<table>
<thead>
<tr>
<th>Position</th>
<th>HSQC (δ\textsubscript{H} ppm)</th>
<th>δ\textsubscript{H} ppm literature*</th>
<th>δ\textsubscript{C} ppm</th>
<th>δ\textsubscript{C} ppm literature*</th>
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<tr>
<td>1</td>
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<td>2</td>
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<td>41.5</td>
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<td>-</td>
<td>213.30</td>
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<tr>
<td>4</td>
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<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>42.16</td>
<td>42.10</td>
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<td>6</td>
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<td>-</td>
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<td>37.40</td>
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<tr>
<td>10</td>
<td>1.52</td>
<td>1.53</td>
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<td>59.50</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>14</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>30.01</td>
<td>30.00</td>
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<tr>
<td>18</td>
<td>1.56</td>
<td>1.56</td>
<td>42.79</td>
<td>42.80</td>
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Discussion
The increasing prevalence of drug resistant TB coupled with the no discovery and bench-to-market development of newer and more potent anti-TB drugs in the past three decades have made it more imperative to search for new and novel anti-TB lead compounds from nature. In this study, the four chromatography fractions NHF1-4 obtained from the n-hexane soluble portion of the 70 % crude ethanol extract from the fruits of *Harungana madagascariensis* were evaluated for anti-TB activity by agar dilution using the LJ-method. Two fractions NHF2 and NHF3 showed bactericidal activity as shown in Table 1. For these two bioactive fractions NHF1 and NHF2, the observed bactericidal effect at the test concentration of 1mg/ml was comparable to that observed for the four standard anti-TB drugs isoniazid, rifampicin, ethambutol and dihydrostreptomycin. This may be due to the bioactive constituents. Further purification of the bioactive fraction NHF2 resulted in the isolation of a creamy to off-white solid. This isolated compound was positive to Liebermann-Burchard test and showed from infra-red (IR) spectroscopy the characteristic aliphatic C-H asymmetric and symmetric stretching vibrations in the region 3002.78 - 2867.64 cm⁻¹ (see Figure 2). Also from the IR spectrum is the evidence of the carbonyl (C=O) stretching vibration at 1713.54 giving a hint that the isolated compound is a triterpene ketone. The one dimensional ¹H-NMR (500 MHz, CDCl₃, δ ppm), ¹³C-NMR (125 MHz, CDCl₃ δ ppm) and DEPT-135 spectra (see Figures 3, 4 and 5) together with the two-dimensional NMR spectra (H-H COSY, HSQC and HMBC see Figures 6, 7 and 8) are evident for eight angular methyl (CH₃), four ring methine (CH), eleven ring methylene (CH₂), six ring quaternary carbon (>C<) and one carbonyl (C=O) as rationalized in Table 2. These spectra data confirmed the chemical structure to be that of the known bioactive triterpene ketone friedelan-3-one with the observed spectra data being in agreement with previous literature report on the phytochemistry of the fruits [8].

Friedelan-3-one (Synonyms: friedelin) is a saturated pentacyclic triterpene ketone [9]. It has been isolated from several plant parts such as of: *Azima tetracantha* [10], *Quercus species* [11], *Terminalia avicennioides* [12], *Harungana madagascariensis* [8] and *Malus domestica* L. [13] among others. Friedelin has been reported to possess many biological
activities such as antifeedant and anti-inflammatory activities [14], hepatoprotective, antimicrobial including antimycobacteria activities [15].

Figure 2 Infra-red spectrum of freidelan-3-one isolated from the fruits of Harungana madagascariensis Lam. Ex. Poiret

Figure 3 $^1$H-NMR spectrum of freidelan-3-one isolated from the fruits of Harungana madagascariensis Lam. Ex. Poiret
Figure 4 13C-NMR spectrum of freidelan-3-one isolated from the fruits of *Harungana madagascariensis* Lam. Ex. Poiret.

Figure 5 DEPT-135 NMR spectrum of freidelan-3-one isolated from the fruits of *Harungana madagascariensis* Lam. Ex. Poiret.
Figure 6 H-H-COSY spectrum of freidelan-3-one isolated from the fruits of *Harungana madagascariensis* Lam. Ex. Poiret

Figure 7 HSQC spectrum of freidelan-3-one isolated from the fruits of *Harungana madagascariensis* Lam. Ex. Poiret
Friedelin isolated from *Maytenus undata* (Celastraceae) was tested against a range of bacteria and fungi implicated in opportunistic and nosocomial infections [13]. Also reported is its antifungal activity against *C. albicans* with a Minimum Inhibition Concentration (MIC) value of 2.44 μg/ml [16]. The antibacterial activity of friedelin against *E. faecalis* with an MIC value of 0.61 μg/ml has been documented [16-17]. Another study by Ichilo et al., [18] showed that friedelin isolated from *Pterocarpus santalinoides* possess an antimicrobial activity against many microbes including *S. aureus* and with MIC values of 10 μg/ml for MRSA, *H. pylori*, *E. coli* and Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) of 40 μg/ml for *E. coli*; 20 μg/ml for MRSA, *H. pylori* and *C. krusei*; and 10 μg/ml for *S. aureus*, *S. pneumonia* and *C. tropicalis*.

Several reports on the growth inhibition activity of friedelan-3-one against several species of *Mycobacteria* have been reported portraying that friedelin can be optimized as an antimycobacteria drug lead. Friedelin isolated from *Terminalia avicennioides* has been shown to have significant antimycobacterial activity against BCG at 4.9 μg/ml [12] while that from *T. brownii* and *T. laxifloragave* a MIC of 250 μg/ml against *M. smegmatis* [19]. Friedelin from cannabis demonstrated activity against three non-pathogenic species at a MIC of 800 μg/ml [20].

5. Conclusion

This is the first time that the anti-*Mycobacteria tuberculosis* activity of the fruit of *H. madagascariensis* is being reported thus validating its ethnomedicinal use in the treatment of cough with blood sputum [6]. Also, literature reports on the biological activities of the isolated triterpene ketone friedelan-3-one from this plant underscores the potentials of this ethno medicinal plant as a source of lead compounds for the development of anti-TB drugs.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of interest.
References


