



# Antimycobacterial activity of scopoletin from ethanolic extract of *Hymenodictyon floribundum* (Hochst. & Steud.) B.L.Rob. Stem bark

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## ARTICLE INFO

### Article history:

Received 11 May 2022

Revised 14 June 2023

Accepted 21 June 2023

Editor DR B Gyampoh

### Keywords:

Tuberculosis

*Hymenodictyon floribundum*

Bioassay-guided isolation

Antimycobacterial activity

## ABSTRACT

The available tuberculosis (TB) therapies are taken for long time with complex regimens associated with adverse effects and drug-drug interactions. The emergence of multidrug resistant (MDR) and extensively drug resistant (XDR) strains of *Mycobacterium tuberculosis* and HIV co-infections necessitates the need for continued search for new effective anti-TB alternative leads with different mechanisms of action and safety profile. The aim of this study was to isolate and characterize antimycobacterial compounds from *Hymenodictyon floribundum* (Hochst. & Steud.) B.L.Rob. a plant used traditionally in the management of HIV/AIDS-related conditions. Antimycobacterial activity of the plant extracts and its constituents against *Mycobacterium indicus pranii* (MIP) and *Mycobacterium madagascariense* (MM) was tested using a twofold broth micro-dilution technique. Bioassay-guided isolation was used to isolate active compounds. Isolated compounds were also tested for activity against clinical isolates of *Mycobacterium tuberculosis* (*Mtb*). The crude plant extract had antimycobacterial activity and its ethyl acetate fraction was the most active with minimum inhibitory concentration (MIC) values of 97 µg/ml and 197 µg/ml against MIP and MM respectively. Three compounds were isolated from the ethyl acetate fraction; 7-hydroxy-6-methoxycoumarin (**1**), 7,7'-dihydroxy-6,6'-dimethoxy-3,3'-biscoumarin (**2**) and 7,7'-dihydroxy-6,6'-dimethoxy-8,8'-biscoumarin (**3**). Compound **1** had growth inhibitory activity against MIP, MM and *Mtb* with MIC values of 0.2 M against MIP, 0.41 M against MM, 0.78 M against *Mtb*1 and 1.63 M against *Mtb*2. In conclusion, 7-Hydroxy-6- methoxycoumarin exhibited moderate antimycobacterial activity. The findings from this study support the use of *H. floribundum* by traditional health practitioners for treatment of HIV and AIDS-related conditions.

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## Introduction

Tanzania is amongst the 30 high tuberculosis (TB) burden and the 30 high TB/HIV burden countries in the world [18]. TB infections have been significantly increased by the emergence of HIV/AIDS [1,3,5] which has led to difficulties in eradicating it. The management of TB is further complicated by the emergence of multidrug resistant (MDR), extensively drug resistant (XDR) strains of *Mycobacterium tuberculosis* and HIV co-infection [2]. Efforts are therefore needed to develop new anti-TB drugs.

Despite the efforts by the government of Tanzania to provide free anti-retrovirals (ARVs) and anti-TB drugs in all regions and districts there are still people who are using traditional medicines either alone or concomitantly with ARVs and a number of plants have been documented [12–14]. *Hymenodictyon floribundum* (Rubiaceae), locally known as Tsere by the Iraqw community, is amongst plants that are used by traditional healers in Mbulu district, Manyara region for the treatment of HIV/AIDS related conditions [14]. In Ethiopia it is used for treatment of eye infections [11,17], while in Angola the barks are used to treat febrile conditions [8]. To the best of our knowledge there are no reports regarding antimycobacterial activity of this plant. Therefore, the purpose of the present work was to test the crude stem bark extract of *H. floribundum* for antimycobacterial activity and carryout bioassay-guided fractionation to isolate and characterize active compounds.

## Materials and methods

### Solvents and reagents

The chemicals and reagents used include ethanol, dimethylsulphoxide (DMSO), normal saline, distilled water, dichloromethane, ethyl acetate, petroleum ether; methanol, acetone, iodinitrotetrazolium chloride (INT), 7H9 broth media and silica gel. The reagents and chemicals used were of analytical grade purchased from Sigma Aldrich and Lab Equip Ltd Dar es Salaam, Tanzania.

### Plant collection and extraction

The stem bark of *H. floribundum* were collected from Mbulu district, Manyara region by Mr Selemani Haji, a botanist from the Department of Botany, University of Dar es Salaam. Voucher specimens (AM & SH1, AM & SH2, AM & SH3) are deposited at the Herbarium of the Institute of Traditional Medicine (ITM), Muhimbili University of Health and Allied Sciences (MUHAS). The samples were dried under shade for two weeks and ground into fine powder. The ground stem bark powder (2.5 Kg) was extracted repeatedly (3 times) with 80% ethanol using maceration method at room temperature for 48 hrs. Later, the mixture was filtered through filter paper (Whatman No. 1, England) in normal glass funnel. The combined crude extract was concentrated using a rotary evaporator at 40 – 50 °C. The dry extract was stored in a refrigerator at –4 °C until when needed for experiment.

### Bioassay-guided fractionation of crude extract

**Fractionation of the crude extract.** During fractionation process, 280 g of the crude extract was dissolved in ethanol, and the resulting solution was mixed with 280 g of silica gel (70–230 mesh). The mixture was concentrated by rotary evaporator to complete dryness and then ground to a fine powder. A total of 480 g of fresh silica gel was packed in a Büchner funnel, followed by the fine crude extract powder mixed with silica gel, and finally followed by small amount of fresh silica gel at the top. Elution was carried out using three solvents of increasing polarity starting with dichloromethane, ethyl acetate and finally ethanol. Three fractions were obtained: dichloromethane fraction ~52.0 g, ethyl acetate fraction ~24.0 g and ethanol fraction ~35.0 g. Each fraction was tested for antimycobacterial activity against MIP and MM. The most active fraction was ethyl acetate, therefore it was chosen for bioassay-guided isolation of active compounds, Table 1.

**Table 1**  
Antimycobacterial activity of the crude extract, fractions and isolated compound against MIP and MM.

| Name of sample              | Mean MIC± SD (µg/ml)<br>MIP (n = 6) | Mean MIC± SD (µg/ml)<br>MM (n = 6) |
|-----------------------------|-------------------------------------|------------------------------------|
| 80% Ethanolic crude extract | 195.00 ± 68.00                      | 781.25 ± 72.00                     |
| DCM fraction                | 391.00 ± 78.00                      | 1562.50 ± 81.00                    |
| Ethyl acetate fraction      | 97.00 ± 0.00                        | 195.00 ± 57.00                     |
| Ethanol fraction            | 192.00 ± 75.00                      | 391.00 ± 85.00                     |
| 1*                          | 0.20 ± 0.059                        | 0.41 ± 0.00                        |
| 2% DMSO                     | No inhibitory effect                | No inhibitory effect               |
| Ciprofloxacin*              | < 0.012± 0.00                       | < 0.012± 0.00                      |

\*Mean MIC in M.

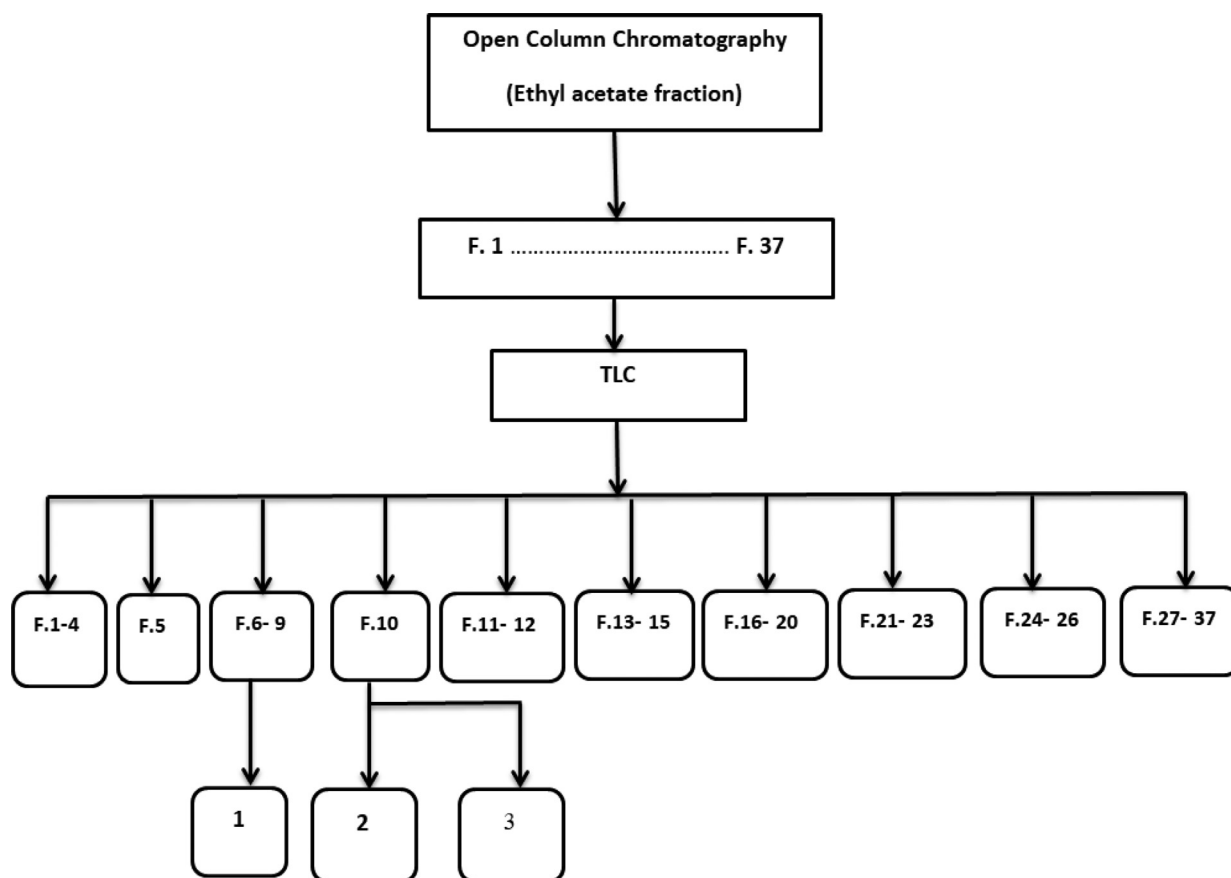


Fig. 1. Schematic diagram showing isolation process.

**Isolation of bioactive compounds.** About 160 g of silica gel 60 (0.04 - 0.063 mm) was suspended in petroleum ether to form a slurry and then transferred into an open column. About 16 g of the dried sample of ethyl acetate fraction was first dissolved in a minimal amount of ethyl acetate solvent and then silica gel added to form a slurry which was allowed to evaporate to dryness, ground into powder and spread on the top of the packed silica gel in the column. Another small amount of silica gel was placed on the surface of the column followed by a piece of cotton wool so as to prevent any disturbance of the packed column during solvent delivery. The column was successively eluted with different ratios of PE/DCM, DCM, and DCM/MeOH to give thirty seven sub fractions of 200 ml each (HF1.2.1–HF1.2.37). Based on TLC profile similar fractions were combined and combination was as follows; F. 1- 4, F. 6- 9, F. 11- 12, F. 13- 15, F. 16- 20, F. 21- 23, F. 24- 26 and F. 27- 37 (Fig. 1). The isolation was monitored by TLC and visualization was accomplished by UV at 254 and 366 nm and acidified vanillin. The combined fraction F.6 - 9 was subjected to small column chromatography for further isolation and yielded compound **1** which was yellow in colour (300.0 mg). Fraction F.10 was also subjected to small column chromatography and yielded compound **2** (3.0 mg) and compound **3** (3.0 mg).

#### Antimycobacterial activity testing

**Tested microorganism.** *Mycobacterium madagascariense* (DSM 44,641) and *Mycobacterium indicus pranii* (DSM 45,239) supplied by DSMZ – the Germany Resource Centre for Biological Materials (Braunschweig, Germany) were obtained from the Department of Biological and Pre-clinical Studies at Institute of Traditional Medicine (ITM), Muhimbili University of Health and Allied Sciences (MUHAS). The clinical isolates of *M. tuberculosis* were obtained from the Tanzanian Central Tuberculosis Reference Laboratory (CTRL).

**Preparation of media and inoculums.** The Middlebrooks 7H9 broth media base was prepared according to manufacturer instructions. Sterile wire loop was used to take some few colonies of mycobacteria into vials containing sterile distilled water and emulsified on the sides of the vial to make a suspension equivalent to 0.5 McFarland turbidity.

**Preparation of stock solution.** The stock solution of dried crude extract, dichloromethane, ethyl acetate, and ethanol fraction and **1** were separately prepared by using the respective broth and solubilization was aided by 2% DMSO. Dilutions were made to set the concentration in the first wells of the microtitre plates at 3.125 µg/ml.

**Table 2**Antimycobacterial activity of the isolated compound against clinical isolates of *M. tuberculosis*.

| Compound Code | MIC $\pm$ SD (M)<br>Mtb1 (n = 6) | Mtb2 (n = 6)       |
|---------------|----------------------------------|--------------------|
| <b>1</b>      | 0.78 $\pm$ 0.048                 | 1.63 $\pm$ 0.048   |
| 2% DMSO       | No inhibition                    | No inhibition      |
| Ciprofloxacin | < 0.012 $\pm$ 0.00               | < 0.012 $\pm$ 0.00 |

**Antimycobacterial testing of the crude extract and the fractions.** Antimycobacterial activity was determined by using 2 fold broth micro-dilution technique using 96 well polystyrene microtiter plates [6,7]. Briefly, the antimycobacterial activity of the crude extract and the fractions was tested against MM and MIP. A volume of 50  $\mu$ L of broth was added into each of the 96 wells using a multichannel pipette with sterilized tips. Equal volumes (50  $\mu$ L) of crude test extract solution was added to the first row (row A) in duplicate, followed by dichloromethane fraction, ethyl acetate fraction, ethanol fraction, negative control and the last two wells were filled by the positive control. Fifty microliters (50  $\mu$ L) were then drawn from each mixed well in row A and transferred to the corresponding row B wells and mixed. The procedure was repeated until the last row H where 50  $\mu$ L for each of the column were discarded leaving only 50  $\mu$ L. For each microorganism the activated suspension was added in a clean and sterilized petri dish and then 50  $\mu$ L were transferred into each of the 96 wells on the plate and then incubated for 24 h. MM was incubated at 31 °C while MIP was incubated at 37 °C. Two hours before reading the result, 40  $\mu$ L of the indicator (0.2% iodinitrotetrazolium) was added into each well and the plates were incubated for 1 hour. The plates were read visually whereby pink/purple colour indicated bacterial growth. Ciprofloxacin was used as a positive control.

**Antimycobacterial testing of isolated compounds.** A twofold broth micro dilution technique was also used to evaluate bioactivity of **1** against MM, MIP and clinical isolates of *M. tuberculosis* to determine its MICs. However, **2** and **3** were not tested due to insufficient amount obtained. With regards to *M. tuberculosis* the plates were incubated for four weeks at 37 °C.

**Spectroscopic analysis of isolated pure compounds.** The structures of all compounds were elucidated by 1D (<sup>1</sup>H and <sup>13</sup>C) and 2D NMR. All spectra were recorded in deuterated solvents in 5 mm tubes at room temperature on a Bruker AVANCE NEO 400 MHz spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany), with the deuterium signal of the solvent as the lock and TMS (for <sup>1</sup>H) or the solvent (for <sup>13</sup>C) as internal standard. To obtain the spectra all raw data were processed with Top Spin Software (Bruker).

## Results

### Antimycobacterial activity

The MICs of the crude ethanol extract of the tested fractions were 195  $\mu$ g/ml and 781.25  $\mu$ g/ml against MIP and MM. The ethyl acetate fraction had the highest activity with MIC of 97  $\mu$ g/ml and 197  $\mu$ g/ml against MIP and MM. Compound **1** was active on both microorganisms with MIC of 0.2 M and 0.41 M against MIP and MM respectively Table 1.

Compound **2** and **3** were isolated in insufficient quantities and therefore, were not tested for antimycobacterial activity. Compound **1** had moderate activity against the two *M. tuberculosis* clinical isolates with MIC of 156  $\mu$ g/ml and 312.5  $\mu$ g/ml against the two isolates used; *Mtb1* and *Mtb2*, Table 2.

### Characterization of isolated compound

In this study three compounds were isolated and their spectroscopic data (**Supplementary material**) were found to be in agreement with literature reports. They were identified as 7-Hydroxy-6- methoxycoumarin (**1**) [19], 7,7'-dihydroxy-6,6'-dimethoxy-3,3'-biscoumarin (**2**), and 7,7'-dihydroxy-6,6'-dimethoxy-8,8'-biscoumarin (**3**) [10,16], Fig. 2.

## Discussion

The ethanol extract of *H. floribundum* stem bark exhibited significant antimycobacterial activity, which was higher against MIP compared to MM. The ethyl acetate fraction of the 80% ethanol extract showed the strongest antimycobacterial activity against both MIP and MM with MIC value of 97.00  $\mu$ g/ml and 195.00  $\mu$ g/ml respectively. Compounds **1**; 7-hydroxy-6-methoxycoumarin (Scopoletin) was tested and showed activity against both MIP and MM with mean MIC value of 0.2 and 0.41 M, respectively as well as mean MIC value of 0.78 M and 1.63 M against two *Mtb* clinical isolates. However, the MIC for **1** is inferior to that of standard anti-TB drugs including pyrazinamide, Rifampicin, isoniazid, ethambutol, streptomycin and Amikacin [20]. A previous study reported that scopoletin isolated from *Pelargonium sidoides* exhibited higher activity

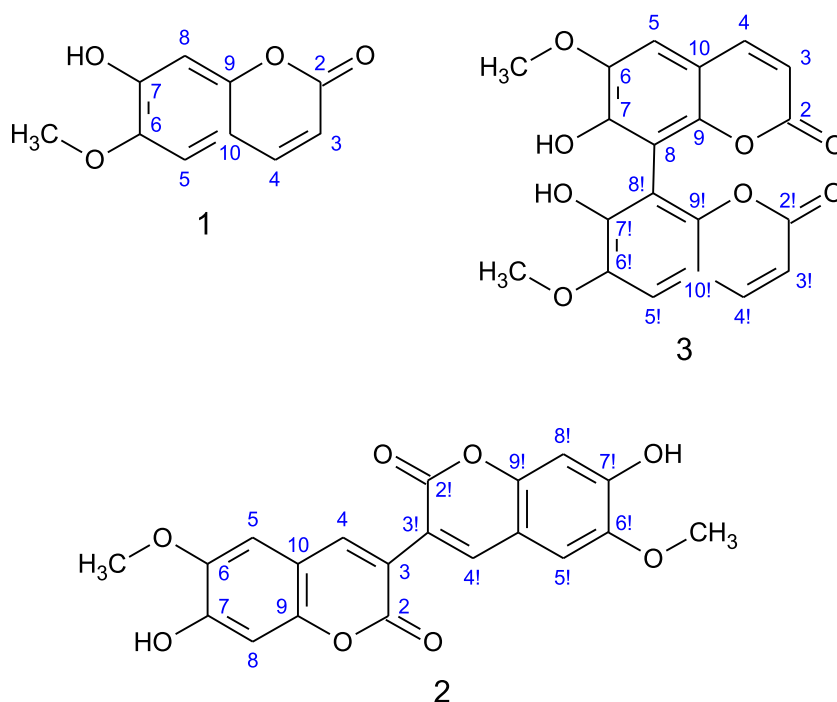


Fig. 2. Structures of compounds (1–3) isolated from *H. floribundum* stem bark extract.

against *Mycobacterium smegmatis* with MIC of 7.8  $\mu\text{g/ml}$  and other bacterial species such as *Escherichia coli*, *Bacillus cereus*, *Staphylococcus intermedius*, and *Listeria monocytogenes*, which is higher than what is reported in this study [15,19].

Scopoletin has also been isolated from various other plants including *Morinda citrifolia* L, *Helichrysum italicum*, *Convolvulus pluricaulis*, *Fatoua pilosa*, *Artemisia annua* and *Lasianthus lucidus* *Morus alba* L [9]. Scopoletin isolated from *Fatoua pilosa* was reported to be active against a **standard mycobacteria strain H<sub>37</sub>Rv** with MIC of 42.1  $\mu\text{g/ml}$  [4]. Apart from antitubercular activity, scopoletin has also been reported to have other several pharmacological activities including antihepatotoxicity, antibacterial, antithyroid, antifungal, antimigratory, antihypertensive, antioxidant, antiproliferative, antiinflammatory, neurological, antidopaminergic, antiadrenergic, antihyperuricemic and antidiabetic activity [9]. The high number of reported biological activities of this constituent of *H. floribundum* supports the use of this plant in the management of HIV and AIDS-related conditions reported previously [14].

Compound 2 and 3 are symmetrical biscoumarins identified as 7,7'-dihydroxy-6,6'-dimethoxy-8,8'-biscoumarin and 7,7'-dihydroxy-6,6'-dimethoxy-3,3'-biscoumarin, respectively. To our knowledge this is the first time biscoumarins are isolated from the genus of *Hymenodictyon* but the same compounds have been isolated from other plant genus [10,16]. Additionally, there is lack of information regarding antimycobacterial activity of these compounds. Unfortunately, the amounts of the compounds isolated were not enough for antimycobacterial activity screening and therefore, there is still a need to screen the compounds for antimycobacterial activity.

## Conclusion

This study has shown that, 80% ethanol extract of the stem bark of *H. floribundum*, its ethyl acetate fraction and the isolated compound exhibited antimycobacterial activities against MM, MIP and clinical isolates of *M. tuberculosis*. The findings support the use of *H. floribundum* by traditional health practitioners for the treatment of cough, tuberculosis and chest infections. However, there is still a need to further explore other constituents of the plant for antimycobacterial activity and toxicity. The future plan is to conduct structure-activity relationship (SAR) studies to modify the chemical structure of scopoletin and identify key functional groups responsible for its antimycobacterial activity so as to enhance the potency. Furthermore, to conduct detailed studies to understand the mechanism of action of the modified scopoletin against drug sensitive *Mycobacterium tuberculosis*, multidrug resistant (MDR) and extensively drug resistant (XDR) strains of *Mycobacterium tuberculosis* using both *in vitro* and *in vivo* assays.

## Data availability statement

Data will be made available on request.

## CRedit authorship contribution statement

**Alphonse Ignace Marealle:** Conceptualization, experimentation, and drafting the initial manuscript. **Alfredi Alfred Moyo:** Conceptualization, experimentation and drafting the initial manuscript. **Francis Machumi:** Interpretation of NMR spectra and reviewing manuscript. **Michael Qwarse:** Experimentation and reviewing manuscript. **Yona Malimi Chenyambuga:** Experimentation and reviewing manuscripts. **Matthias Heydenreich:** Interpretation of NMR spectra and reviewing manuscript. **Mainen Moshi:** Conceptualization, experimentation and reviewing manuscript.

## Declaration of Competing Interest

The authors declare no conflict of interest.

## Acknowledgments

We are grateful to the Central Tuberculosis Reference Laboratory for allowing us to use their facility. Thanks to Mr. Gustav Pius Moyo and Mrs. Mwajuma Moyo for their financial support.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.sciaf.2023.e01778](https://doi.org/10.1016/j.sciaf.2023.e01778).

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