Antimycobacterial, Antibacterial and Antifungal Activities of the Methanolic Extract and Compounds from *Garcinia Polyantha*

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Summary

The crude methanolic extract from the stem bark of *Garcinia polyantha* (GPB), 5-hydroxyflavone (**1**), 1,5-dihydroxyxanthone (**2**), Bangangxanthone A (**3**), 1,3,5-trihydroxyxanthone (**4**) and 1,3,6,7-tretrahydroxyxanthone (**5**) isolated from this extract, were tested for their antimycobacterial activity against a non pathogenic *Mycobacterium smegmatis*, and on *Mycobacterium tuberculosis* using the micro-dilution and radiometric BACTEC respiratory technique respectively. The antibacterial activity against Gram-positive and negative bacteria and the antifungal activity of the above samples were also investigated. The results showed that the crude extract as well as some of the isolated compounds was active on the two tested mycobacterial species. Compound **5** showed the best activity on the two tested mycobacterial species with a recorded Minimal inhibition concentration (MIC) of 4.88 μg/ml. The results obtained also showed that GPB was active on all the 17 tested microorganisms, including nine Gram positive and four Gram-positive bacteria, and four fungi. The overall results of this study provided promising baseline information for the potential use of the studied plant in the treatment of tuberculosis, infections associated with fungi, Gram positive and Gram-negative bacteria.

Keywords: *Garcinia polyantha*, Guttiferae, compounds, antimicrobial activity

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Introduction

The importance of infection cause by *M. tuberculosis*, fungi such as Candida albicans as well as problematic Gram-positive *Staphylococcus aureus* and Gram-negative *Pseudomonas aeruginosa* amongst all is not yet to be demonstrated. Tuberculosis (TB) is the leading killer among all infectious diseases worldwide. The increase in multi-drug resistance in recent times also resulted in increase of victims of Tb and other infectious diseases (1). Therefore, the search for new antimicrobial agents has become increasingly important. Plant kingdom is known to be a potential source of antimicrobial drugs. The Garcinia plants (Clusiaceae), have a wide range of biological properties such as cytotoxic, anti-fungal, anti-microbial, anti-oxidant, anti-inflammatory, anti-HIV activities (2-5). Xanthones and flavonoids were found to be the major compounds associated with the therapeutic potential of *Garcinia* species (2). *Garcinia polyantha* Oliv. is one of the most important medicinal plants belonging the family Guttiferae. In the West and Central Africa, the yellow resinous sap (latex) of *G. polyantha* is used to make a dressing for wounds (6). Despite the various uses of this plant in traditional medicine, no scientific report has been focused on the biological activity of *G. polyantha*. The aim of this study was therefore to investigate the antimycobacterial, the antibacterial and the antifungal activities of the stem bark of *Garcinia polyantha*.

Methods

Plant material

The stem bark of *Garcinia polyantha* Oliv. was collected from Mount Kala ,Center province of Cameroon during May 2004. A specimens (21337/SRF/Cam) is maintained at National Herbarium of Cameroon where the plant was identified.

Purification and General procedures

The air-dried and powdered stem bark (4 Kg) of *G. polyantha* was extracted with CH_2Cl_2/MeOH (1:1) (12 L) at room temperature for 48 h. The filtrate was concentration under reduce pressure to give 85 g of crude extract (GPB)
Eighty grams (80 g) of extract were subjected to column chromatography on silica gel 60 using hexane/CH_2Cl_2 gradient as eluent. Three main fraction named A_0 (4 g), B_0 (18 g) and C_0 (42 g) were obtained following TLC analysis. The purification of A_0 over silica gel column eluting with hexane/CH_2Cl_2 (50:50) afforded 5-hydroxyflavone $C_{15}H_{10}O_3$ (**1**; yellow oil; 112 mg; $M_{w:}$ 238; Rf 1.9 with hexane/CH_2Cl_2 60:40) (7). Fraction B_0 under similar conditions yielded 1,5-dihydroxyxanthone $C_{13}H_8O_4$ (**2**; yellow powder; 136 mg; $M_{w:}$ 228; Rf 2.6 with CH_2Cl_2) (8), and Bangangxanthone A $C_{18}H_{16}O_5$ (**3**; yellow needle crystals; 73 mg; $M_{w:}$ 312; Rf 2.3 with CH_2Cl_2) (9). The purification of fraction C_0 similarly of A_0 afforded 1,3,5-trihydroxyxanthone $C_{13}H_8O_5$ (**4**; yellow powder; 103 mg; $M_{w:}$ 244; Rf 1.6 using CH_2Cl_2/acetone 95:5) (10). Compound **3** (35 mg) and 1,3,6,7-tetrahydroxyxanthone $C_{13}H_8O_6$ (**5**; yellow powder; 93 mg; $M_{w:}$ 260; Rf 1.0 using CH_2Cl_2/acetone 85:15) (11). The chemical structures of compounds **1**-**5** are illustrated on Figure 1.

Aluminium sheet pre-coated with silica gel 60 $F_{254 nm}$ (Merck) was used for thin layer chromatography and the isolated spots were visualized using both ultra-violet light (254 and 366 nm) and 50% H_2SO_4 spray reagent. The chemical structure of each of the isolated compound was determined on the basis of spectral data produced by one and two-dimensional nuclear magnetic resonance (NMR), recorded on Brüker DRX-400 instrument. This spectrometer was equipped with 5-mm, ^{1}H- and ^{13}C-NMR probes operating at 400 and

100 MHz, with tretramethylsilane (TMS) as internal standard. Mass spectra were recorded on an API QSTAR pulsar mass spectrometer.

Antimicrobial assays

Microbial strains

Mycobacterium smegmatis (MC^2 155) and a drug-susceptible strain of *M. tuberculosis* H37Rv (ATCC 27264) were used. The strains were obtained from American Type, MD, USA Culture Collection and the antimycobacterial tests were preformed at the Department of Plant Science, University of Pretoria, South Africa. Other microbial strains used in this study include *Bacillus cereus* LMP0404G, *Bacillus subtilis* LMP0304G, *Staphylococcus aureus* LMP0206U, *Streptococcus faecalis* LMP0207U (Gram-positive bacteria), *Escherichia coli* LMP0101U, *Shigella dysenteriae* LMP0208U, *Proteus mirabilis* LMP0504G, *Klebsiella pneumoniae* LMP0210U, *Pseudomonas aeruginosa* LMP0102U, *Salmonella typhi* LMP0209U, *Morganella morganii* LMP0904G, *Citrobacter freundii* LMP0804G, *Enterobacter cloacae* LMP1104G (Gram-negative bacteria), *Candida albicans* LMP0204U, *Candida gabrata* LMP0413U, *Microsporum audouinii* LMP0725D, *Trichophyton rubrum* LMP0723D (fungi). *Bacillus cereus* was provided by the A.F.R.C Reading Laboratory of Great Britain. Other strains were clinical isolates from '*Centre Pasteur du Cameroon*', Yaoundé. They were maintained on agar slant at 4°C in the Laboratory of Applied Microbiology and Molecular Pharmacology (LMP) (Faculty of Science, University of Yaoundé I) where the tests were conducted. These strains were sub-cultured on a fresh appropriate agar plate 24 hours prior to any antimicrobial test.

Antimicrobial assays

Culture media

    M. smegmatis was cultured onto Middlebrook 7H11 agar (7H11) and allowed to grow for 24 h. *M. tuberculosis* was plated onto Löwenstein-Jensen medium and allowed to grow for 3 - 4 weeks at 37°C. The 7H9 broth and the 7H12 medium containing ^{14}C-labelled substrate (palmitic acid) were used to determine the MIC and the MBC of the test samples on *M. smegmatis* and *M. tuberculosis.* Nutrient Agar (NA) containing Bromocresol purple was used for the activation of *Bacillus* species while NA was used for other bacteria. Sabouraud Glucose Agar was used for the activation of the fungi. Nutrient broth containing 0.05% phenol red and supplemented with 10% glucose (NBGP) was used for MIC and Minimal Microbicidal Concentration (MMC) determinations on microorganisms other than *Mycobacterium sp*. The Mueller Hinton Agar (MHA) was also used for the determination of the MMC on these species.

Chemicals for antimicrobial assay

Ciprofloxacin and isoniazid (INH) (Sigma-Aldrich Chemical Co., South Africa) were used as positive control for *M. smegmatis* and *M. tuberculosis* respectively. Nystatin (Maneesh Pharmaceutic PVT. Ltd., Govandi, Mumbai, 400 043 India) and Gentamicin {Jinling Pharmaceutic (Group) corp., Zhejang Tieng Feng Pharmaceutic Factory, No. 11 Chezhan Road, Huzhou city, Zhejang, China) were used as reference antibiotics (RA) respectively against yeasts and other bacteria than *M. smegmatis* and *M. tuberculosis*.
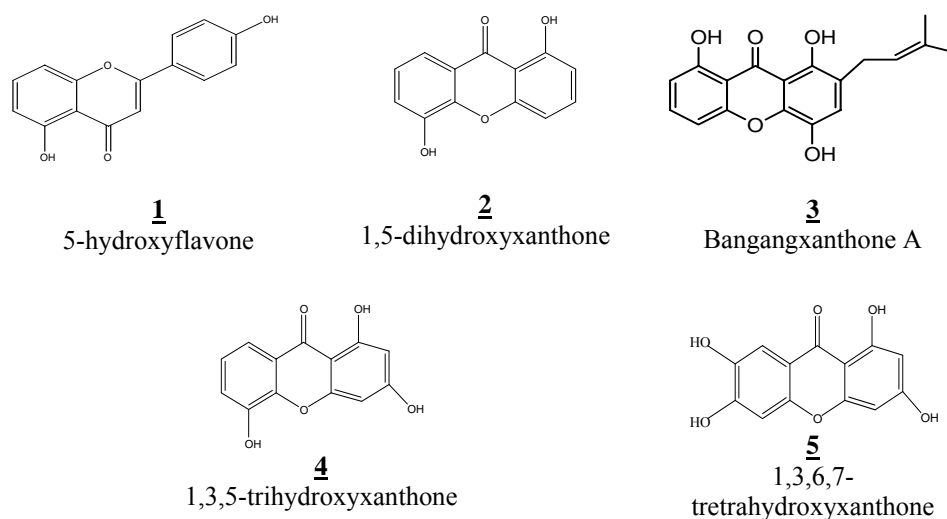
1
5-hydroxyflavone

2
1,5-dihydroxyxanthone

3
Bangangxanthone A

4
1,3,5-trihydroxyxanthone

5
1,3,6,7-
tretrahydroxyxanthone

Figure1. Chemical structures of compounds isolated from *G. polyantha*

Antimycobacterial assays

Microplate susceptibility testing against M. smegmatis.

All samples were tested against *M. smegmatis* using microplate dilution method. The MIC, MBC and bacteria preparation were performed in 96-well microplates according to Salie et al. (12) and Newton et al. (13). The samples were dissolved in 10% dimethylsulfoxide (DMSO) in sterile 7H9 broth to obtain a stock concentration of 1.250 mg/ml. Serial two-fold dilutions of each sample to be evaluated were made with 7H9 broth to yield volumes of 100 μl/well with final concentrations ranging from 0.31-312.50 μg/ml. Ciprofloxacin served as the positive drug control. 100 μl of *M. smegmatis* (0.2 log-phase, yielding 1.26 x 10^8 CFU/ml) was also added to each well containing the sample and mixed thoroughly to give final concentrations ranging from 0.31-19.53 μg/ml. The solvent control, DMSO at 2.5% or less in each well did not show inhibitory effects on the growth of the *M. smegmatis*. Tests were done in triplicates. The cultured microplates were sealed with parafilm and incubated at 37°C for 24 h. The MIC of samples was detected following addition (40 μl) of 0.2 mg/ml *p*-iodonitrotetrazolium chloride (INT, Sigma-Aldrich, South Africa) and incubated at 37°C for 30 min (14). Viable bacteria reduced the yellow dye to a pink colour.
MIC was defined as the lowest sample concentration that prevented this change and exhibited complete inhibition of bacterial growth. The MBC was determined by adding 50 μl aliquots of the preparations (without INT), which did not show any growth after incubation during MIC assays, to 150 μl of 7H9 broth. These preparations were incubated at 37°C for 48 h. The MBC was regarded as the lowest concentration of extract, which did not produce a colour change after addition of INT as above mentioned.

Antitubercular rapid radiometric assay using M. tuberculosis.

The radiometric respiratory technique using the BACTEC 460 system (Becton Dickinson Diagnostic Instrument, Sparks, md) was used for susceptibility testing against *M. tuberculosis* as described previously (15). Solutions of all the samples were prepared in Dimethylsulfoxide (DMSO) to obtain a stock concentration of 4.00 mg/ml. Control experiments showed that a final concentration of DMSO (1%) in the medium had no adverse effect on the growth of *M. tuberculosis*. The primary drug INH served as drug-control. A homogenous culture (0.1 ml of *M. tuberculosis*, yielding 1×10^4 to 1×10^5 CFU/ml), was inoculated in the vials containing sample as well as in the control vials (16). The tested concentrations ranged from 0.012-312.50 µg/ml. Three sample-free vials were used as controls (medium + 1% DMSO); two vials (V1) were inoculated in the same way as the vials containing the sample, and one (V2) was inoculated with a 1:100 dilution of the inoculum (1:100 control) to produce an initial concentration representing 1% of the bacterial population (1×10^2 to 1×10^3 CFU/ml).

Mycobacterium growing in 7H12 medium containing ^{14}C-labelled substrate (palmitic acid) uses the substrate and produced $^{14}CO_2$. The amount of $^{14}CO_2$ detected is expressed in terms of the growth index (GI) (17). Inoculated bottles were incubated at 37°C and each bottle was assayed everyday to measure the GI. Therefore, the MIC was considered as the lowest concentration inhibiting more than 99% of the initial bacterial population. This was taken as the lowest concentration of the sample with recorded ΔG lower than that of 1:100 control (15)

Antimicrobial assay on Gram-positive, Gram-negative bacteria and fungi

Sensitivity test: agar disc diffusion assay

Preparation of discs

Whatmann filter paper (N°1) discs of 6 mm diameter were impregnated with 10 µl of the solution of crude extract at 20 mg/ml (200 µg/disc) and isolated compounds at 10 mg/ml (100 µg/disc) prepared using DMSO. The discs were evaporated at 37°C for 24 hours. The RA discs (Gentamicin for bacteria and nystatin for fungi) were prepared as described above using the appropriate concentrations to obtain discs containing 20 µg of drug. Two discs were prepared for each sample.

Diffusion test

The antimicrobial diffusion test was carried out as previously described by (18) using a cell suspension of about 1.5×10^6 CFU/ml obtained from a McFarland turbidity standard N° 0.5. The suspension was standardised by adjusting the optical density to 0.1 at 600 nm (SHIMADZU UV-120-01 spectrophotometer) (18). This was used to inoculate by flooding the surface of MHA plates. Excess liquid was air-dried under a sterile hood and the impregnated discs applied at equidistant points on top of the agar medium. A disc prepared with only the corresponding volume of DMSO was used as negative control. The plates were incubated at 37°C for 24 hours. Antimicrobial activity was evaluated by measuring the

Table1
 Antimycobacterial activity of the crude extracts, compounds isolated from *G. polyantha* and reference antibiotics

Tested samples*	*M. smegmatis*		*M. tuberculosis*	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	ΔG
Crude extract	78.12	156.25	78.12	-7.25±1.15
1	-	-	-	-
2	78.12	312.50	-	-
3	39.06	78.12	39.06	2.50±0.12
4	78.12	312.50	-	-
5	4.88	39.06	4.88	-1.00±2.83
RA	0.61	1.22	0.12	-6.50±0.72

* The Tested samples were crude extract from *G. polyantha:* 5-hydroxyflavone (**1**); 1,5-dihydroxyxanthone (**2**), Bangangxanthone A (**3**), 1,3,5-trihydroxyxanthone (**4**), 1,3,6,7-tretrahydroxyxanthone (**5**); Ciprofloxacin and isoniazid as reference antibiotics or RA for *M. smegmatis* and *M. tuberculosis* respectively.
(-) : not determined as MIC>312.50 µg/ml

diameter of the inhibition zone (IZ) around the disc. The assay was repeated twice and the results expressed using signs as follows: (-) for no activity and (+) for samples with IZ >6 mm.

MIC and MMC determinations

The MICs of test samples and RA were determined as follows: the test sample was first of all dissolved in Dimethylsulfoxide (DMSO). The solution obtained was added to NBGP to a final concentration of 156.25 12 µg/ml for the crude extracts and 78.12 µg/ml for the isolatedcompounds and RA. This was serially diluted two fold to obtain concentration ranges of 2.44 to 156.25 µg/ml for the crude extracts and 0.61 to 78.12 µg/ml. 100 µl of each concentration was added in a well (96- wells microplate) containing 95 µl of NBGP and 5 µl of inoculum (standardised at $1.5 \ 10^6$ CFU/ml by adjusting the optical density to 0.1 at 600 nm SHIMADZU UV-120-01 spectrophotometer. The final concentration of DMSO and Tween in the well was less than 1% (preliminary analyses with 1% (v/v) DMSO/NBGP and 10% v/v Tween 20/NBGP affected neither the growth of the test organisms nor the change of color due to this growth). The negative control well consisted of 195µl of NBGP and 5µl of the standard inoculum (18). The plates were covered with a sterile plate sealer, then agitated to mix the content of the wells using a plate shaker and incubated at 37°C for 24 hours. The assay was repeated twice. Microbial growth was determined by observing the change of color in the wells (red when there is no growth and yellow when there is growth). The lowest concentration showing no color change was considered as the MIC.
For the determination of MMC, a portion of liquid (5µl) from each well that showed no change in color was plated on MHA and incubated at 37°C for 24 hours. The lowest concentration that yielded no growth after this sub-culturing was taken as the MMC (18).

Results

The compounds isolated from the stem barks of *G. polyantha* were 5-hydroxyflavone (**1**), 1,5-dihydroxyxanthone (**2**), Bangangxanthone A (**3**), 1,3,5-trihydroxyxanthone (**4**), 1,3,6,7-

Table2
Antimicrobial activity of the crude extracts, compounds isolated from *G. polyantha* and reference antibiotics

Microorganisms	Diffusion Test				Minimum inhibition concentration (µg/ml)				Minimum microbicidal concentration (µg/ml)			
	GPB	**1**	**5**	RA	GPB	**1**	**5**	RA	GPB	**1**	**5**	RA
Gram negative bacteria												
Citrobacter freundii	+	+	+	+	78.12	39.06	nd	9.76	nd	78.12	nd	19.53
Enterobacter cloacae	+	-	-	+	78.12	-	-	9.76	156.25	-	-	19.53
Escherichia coli	+	-	+	+	39.06	-	nd	4.88	78.12	-	nd	9.76
Klebsiella pneumoniae	+	+	+	+	78.12	nd	nd	2.44	nd	nd	nd	4.88
Morganella morgani	+	+	+	+	39.06	nd	nd	2.44	78.12	nd	nd	4.88
Proteus mirabilis	+	+	+	+	39.06	nd	nd	2.44	78.12	nd	nd	4.88
Pseudomonas aeruginosa	+	-	+	+	39.06	-	9.76	9.76	156.25	-	39.06	19.53
Shigella dysenteriae	+	+	+	+	19.53	39.06	nd	2.44	39.06	78.12	nd	4.88
Salmonella typhi	+	+	+	+	78.12	nd	nd	2.44	156.25	nd	nd	4.88
Gram positive bacteria												
Streptococcus faecalis	+	-	-	+	39.06	-	-	9.76	78.12	-	-	19.53
Staphylococcus aureus	+	+	+	+	78.12	nd	9.76	2.44	156.25	nd	39.06	4.88
Bacillus cereus	+	-	+	+	39.06	-	nd	4.88	78.12	-	nd	9.76
Bacillus subtilis	+	-	+	+	39.06	-	19.53	4.88	78.12	-	39.06	9.76
Fungi												
Candida albicans	+	+	+	+	156.25	nd	39.06	2.44	nd	nd	nd	4.88
Candida gabrata	+	+	+	+	78.12	39.06	39.06	2.44	nd	78.12	nd	4.88
Microsporum audouinii	+	-	+	+	78.12	-	39.06	1.22	156.25	-	78.12	2.44
Trichophyton rubrum	+	+	-	+	156.25	nd	-	1.22	nd	nd	-	2.44

*The Tested samples were crude extract from), *G. polyantha* (GPB); 5-hydroxyflavone (**1**); 1,3,6,7-tretrahydroxyxanthone (**5**);); RA or Reference antibiotics (Gentamicin for bacteria, Nystatin for yeasts).
(x): Not tested; (+): Active; (-): Not active

tretrahydroxyxanthone (**5**) (Figure 1). The activity of the crude extract, the isolated compounds and RA is reported in Tables 1 and 2. This result indicates that the crude extract from *G. polyantha* is active on *M. smegmatis*. MIC values lower than 50 µg/ml were obtained with compounds **3** and **5**. The lowest MIC of 78.12 µg/ml was noted with the crude extract meanwhile the corresponding value for compounds (4.88 µg/ml) was obtained with **5**. The results of the disc diffusion assay (Table2) indicated that the crude extracts from *G. polyantha* were able prevent the growth of all the tested fungi, Gram-positive and negative bacteria. The tested compounds showed a selective activity. Table2 also showed that, compounds **5** was active on 14 (82.4 %) of the 17 tested organisms and **1** on 10 (58.8 %). The results of the MIC determination summarized on Table2 also confirmed the interesting antimicrobial potency of the crude extracts from this plant. However, MIC values ranged from 19.53 to 156.25 µg/ml were noted with the crude extract from *G. polyantha* on all the microorganisms tested in the diffusion assay. The tested compounds showed an inhibition effect with a detectable MIC on at least one of the tested microorganisms. The results of the MMC determination (Table2) indicated that the MMC values were obtained with the crude extract on 12 of the 17 microorganisms investigated following the diffusion test.

Discussions

The compounds isolated from the stem barks of *Garcinia polyantha* were a known flavonoid, (compound **1**) and four xanthones. The lowest MIC value obtained with the crude extract (39.06 µg/ml) was 128

fold greater than that of Ciprofloxacin. Newton et al. (13) have demonstrated that the sensitivity of *M. tuberculosis* is closer to that of *M. smegmatis,* a non-pathogenic microorganism. Therefore, this pathogen can be used for preliminary study to select compounds with potential activity on *M. tuberculosis*. Furthermore, plant extracts with MIC values lower than 100 µg/ml are generally considered as promising antimicrobial drug (19). We had therefore selected samples with the MIC values lower than 100 µg/ml (compounds **3** and **5**) for the susceptibility test against *M. tuberculosis*. Compound **5** showed the most interesting activity on this pathogen. The MIC obtained with this compound was about 41 fold greater than that of INH. The results obtained in this study corroborate the use of *M. smegmatis* in the pre-screening of anti-tubercular drugs. The results of the MBC determination also indicated that the cidal effect of the tested sample could be expected on *M. tuberculosis*. This suggests that the crude extract as well as compounds **3** and **5** could be useful for the development of anti-tubercular drugs.

The lowest MIC values (19.53 µg/ml) were obtained with the crude extract on *Shigella dysenteriae*. The corresponding value for the tested compounds (9.76 µg/ml) was recorded with compound **5** on *S. aureus* and *P. aeruginosa*. A keen look of the results of both MIC and MMC determinations shows that the obtained MMC values are not more than four fold greater than the MICs in most of the cases. This suggests that cidal effects of the tested samples could be expected on many of the tested microorganisms (20). In our previous study (20), compounds **2**, **3** and **4** isolated from G. *smeathmanii,* another plant from the genus *Garcinia*, were found to be very active on Gram-positive and negative bacteria. The results of this study also confirm the antimicrobial activity of plants from the genus *Garcinia*. The results of the present investigation can be considered as very important, if taking in account the worldwide importance of TB in general, and particularly the severity of this infection amongst the HIV infected population in Africa. These results are also very important, if consider the importance of the tested medical importance of the tested organisms and their sensitivity to the tested samples.

To the best of our knowledge, the antimicrobial activity of the crude extract from *G. polyantha* as well as that of 1,3,6,7-tretrahydroxyxanthone is being reported for the first time. Nevertheless, the inhibition potential of benzophenones and xanthones is not yet to be demonstrated (20-21).

The overall results indicate promising baseline line information for the potential use of the tested extracts as well as some of their components in the treatment of tuberculosis.

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