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Antimiycobacterial, antibacterial and antifungal activities of *Terminalia superba* (Combretaceae)

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Abstract

The methanol extract from the stem bark of *Terminalia superba* (TSB), fractions (TSB1 - 7) and two compounds isolated following bio-assay guided fractionation namely 3,4'-di-O-methylellagic acid 3'-O-β-D-xylopyranoside (**1**) and 4'-O-galloy-3,3'-di-O-methylellagic acid 4-O-β-D-xylopyranoside (**2**) were evaluated for their antimycobacterial, antibacterial and antifungal activities. The broth microdilution, microplate Alamar Blue assay (MABA) and agar disc diffusion methods were used for the investigations. The results of the antimycobacterial assays showed that the crude extract, fraction TSB5-7 and compound **1** were able to prevent the growth of all the studied mycobacteria. The lowest minimal inhibitory concentration (MIC) value of 39.06 µg/ml for this extract was recorded on both *M. smegmatis* and *M. tuberculosis* MTCS2. The corresponding values were 19.53 µg/ml and 4.88 µg/ml for fractions and compounds respectively. The MIC determinations results on other organisms indicate values ranging from 19.53 to 78.12 µg/ml for TSB and compound **2** on 90.9% of the tested organisms, meanwhile compounds **1** as well as fractions TSB 6 and 7 exhibited detectable MIC values on all studied microorganisms.

The overall results provide promising baseline information for the potential use of the crude extract from *Terminalia superba*, fractions 6-7 and the tested compounds in the treatment of tuberculosis, bacterial and fungal infections.

*Keywords***:** *Terminalia superba*; Combretaceae; fractions; compounds; antimicrobial activity.

1. Introduction

The establishment of concrete scientific basis today needs to enable more rational exploitation of African medicinal plants. Plant drugs are widely used in Africa for the treatment of many ailments and constitute the first health recourse for about 80% of the population (Sofowora 1993). A numbers of pharmaceutical products in current use are derived from plants (Cowan 1999). Medicinal plants are rich in compounds which may be potential natural drugs and which may serve as an alternative, cheap and safe antimicrobials for the treatment of common ailments. The present report is focused on *Terminalia superba* Engl. & Diels (Combretaceae), a tree of about 30-50 m high. It is a member of the genus *Terminalia* that comprises around 100 species distributed in tropical regions of the world. *T. superba* is known as *"Akom"* in Cameroon and is locally used in the treatment of various ailments, including diabetes mellitus, gastroenteritis, female infertility and abdominal pains (Adjanohoun et al. 1996). The anti-diabetic activity of the stem bark of *T. superba* on streptozotocin-induced diabetic rats was lately demonstrated (Kamtchouing et al. 2006). Compounds **1** and **2** have previously showed β-Glucosidase inhibitory activity (Tabopda et al. 2008). Therefore, the present work was, undertaken to evaluate the antimycobacterial, antibacterial and antifungal activities of the methanol extract from the stem bark of *Terminalia superba* as well as the fractions and compounds obtained following a bio-assay guided fractionation.

2. Material and methods

2.1. Plant material

The stem bark of *Terminalia superba* Engl. & Diels was collected from Ebolowa, Southern region of Cameroon, in June 2004. The plant was identified at the Cameroon National Herbarium, Yaounde, (by Mr. Victor Nana) where a voucher specimen (W.C.S. 3642a/12543/Ya) was deposited.

2.2. Extraction and bio-assay guided purification

The air-dried, free from endotoxin and powdered stem bark (1.5 Kg) of *T. superba* was extracted by maceration in methanol (6 L) at room temperature for 48 h. The filtrate was then concentrated under vacuum to give a dark crude extract (TSB) (73 g). The extract (55 g) was subjected to a silica gel-60 column chromatography and eluted with hexane-ethyl acetate (hex-EtOAc) and ethyl acetate-methanol (EtOAc-MeOH) gradients. One hundred and forty five fractions of 150 ml each were collected as follows: hex (1-12), hex-EtOAc 75:25 (13-38), hex-EtOAc 50:50 (39-55), hex-EtOAc 25:75 (56-77), EtOAc (78-91), EtOAc-MeOH 95:5 (92-115), MeOH (116-145). These fractions were then pooled following analytic TLC in seven new fractions: TSB1 (1-8; 1.8 g), TSB1 (9-26; 2.1 g), TSB3 (27-42; 3.2 g), TSB4 (43-61; 3.6 g), TSB5 (62-86; 5.4 g), TSB6 (87-106; 11.5 g), TSB7 (107-145; 15.8 g). On the basis of the antimicrobial investigations of the above fractions, TSB6 and TSB7 were each subjected to a second column chromatography. Eight grams of TSB6 were passed through Sep-Pak C_{18}-cartridges (15 g) using H_2O-MeOH and $CHCl_3$ as solvent to yield a grey powder identified as ellagic acid ($C_{14}H_6O_8$) (**3**; 16 mg, *Mw:* 302.20, mp 361-362°C) (Grzegorz and Jaromir, 1996) and a crystal from methanol identified as 4'-*O*-galloy-3,3'-di-*O*-methylellagic acid 4-*O*- β -D-xylopyranoside ($C_{28}H_{22}O_{16}$) (**2**, 56 mg, *Mw:* 613.0824, mp 204-206°C) (Tabopda et al. 2008). TSB7 (10 g) was purified on Sephadex LH-20 (Pharmacia) CC with H_2O/MeOH gradient to yield compound **2** (13 mg) and crystals (from methanol) identified as

3,4'-di-*O*-methylellagic acid 3'-*O*-β-D-xylopyranoside ($C_{21}H_{18}O_{12}$) (**1**, 93 mg, *Mw:* 461.0791, mp 194-196°C) (Tabopda et al. 2008). The chemical structures of the isolated compounds are presented in Fig. 1.

2.3. General procedure

IR spectra were recorded on an ATI Mattson Genesis Series FTIR spectrometer as KBr disc. ^{1}H-NMR, ^{13}C-NMR, two-dimensional COSY, ROESY, HSQC and HMBC analysis were performed on a Bruker Avance DPX instrument (300 MHz and 500 MHz for ^{1}H and 75 MHz for ^{13}C). The 2.50 and 40.0 ppm resonances of residual CD_3SOCD_3 were used as internal references for ^{1}H and ^{13}C-NMR spectra, respectively. Mass spectra were recorded on a micro TOF instrument. All melting points were determined on a micro-melting point apparatus and are uncorrected. The structures of the compounds were confirmed by comparing with reference data from available literature.

2.4. Microbial strains

The tested organisms included: *Mycobacteria* namely; *Mycobacterium smegmatis* ATCC 700084, drug-susceptible strain of *Mycobacterium tuberculosis* H37Rv ATCC 27294 (America Type Culture Collection, Rockville, MD, USA), two clinical strains of *M. tuberculosis* MTCS1, *M. tuberculosis* MTCS2, and a methicillin-resistant *Staphylococcus aureus* LMP805 (Gram-positive bacterium), six Gram-negative bacteria namely; β-lactamase positive *(βL⁺) Escherichia coli* LMP701, βL⁺-*Shigella dysenteriae* LMP606, Ampicillin-resistant *Klebsiella pneumoniae* LMP803, Carbenicillin-resistant *Pseudomonas aeruginosa* LMP804, Chloramphenicol-resistant *Salmonella typhi* LMP706, Chloramphenicol-resistant *Citrobacter freundii* LMP802 and four fungi namely; *Candida albicans* LMP709U, *Candida glabrata* LMP0413U, *Microsporum audouinii* LMP725D and *Trichophyton rubrum* LMP0723D. Each drug to which a microorganism was resistant was used as specific antibiotic (SA) in this study. The clinical isolates were obtained from Yaoundé General Hospital

(Cameroon) and their identity was confirmed before use as previously reported (Mbaveng et al. 2008).

2.5. Culture media

Mycobacterium smegmatis was cultured on Middlebrook 7H11 agar (7H11) and allowed to grow for 24 h. *Mycobacterium tuberculosis* was plated on Löwenstein–Jensen medium and allowed to grow for 3–4 weeks at 37° C. The 7H9 broth was used to determine the minimal inhibitory concentrations (MIC) and the minimal microbicidal concentrations (MMC) of the test samples on *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*. Nutrient Agar (NA) was used for other bacteria. Sabouraud Glucose Agar was used for the activation of the fungi meanwhile the Mueller Hinton broth (MHB) was used to determine the MIC of all samples against the tested pathogens. The MHB and Mueller Hinton Agar (MHA) were used to determine the MMC of the active samples.

2.6. Chemicals

Ciprofloxacin and isoniazid (INH) (Sigma–Aldrich) were used as positive controls for *M. smegmatis* and *M. tuberculosis*. 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) against fungi and bacteria respectively.

2.7. Microplate susceptibility testing against Mycobacterium smegmatis

All samples were tested against *Mycobacterium smegmatis* using the microplate dilution method. The MIC, MMC and bacteria preparation were performed in 96-well microplates according to Salie et al. (1996) and Newton et al. (2002). The crude extract was dissolved in 10% dimethylsulfoxide (DMSO) in sterile 7H9 broth. Serial twofold dilutions of each sample to be evaluated were made with 7H9 broth to yield volumes of 100 µl/well with final concentrations ranging from 0.31-78.12 µg/ml for the crude extract and fractions, 0.16-

39.06 µg/ml for compounds **1** and **2**. Ciprofloxacin served as the positive drug-control. One hundred microlitres of *M. smegmatis* (10^6 CFU/ml) was also added to each well containing the samples and mixed thoroughly at the same concentration range as above. The solvent control; DMSO at 2.5% or less in each well did not show inhibitory effects on the growth of *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 each sample 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 (Eloff, 1998; Mativandlela et al., 2006). Viable bacteria reduced the yellow dye to pink. MIC was defined as the lowest sample concentration that prevented this change and exhibited complete inhibition of bacterial growth. The MMC 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 MMC was regarded as the lowest concentration of extract, which did not produce a color change after addition of INT as mentioned above.

2.8. Antituberculosis Activity: MABA susceptibility testing

The activity of all samples against *M. tuberculosis* was tested using the microplate Alamar Blue assay (MABA) according to Collins and Franzblau (1997), as modified by Jimenez-Arellanes et al. (2003). Briefly, each of the above strains was cultured at 37°C in Middlebrook 7H9 broth (Becton Dickinson, Sparks, MD) supplemented with 0.2% glycerol (Sigma Chemical Co., St. Louis, MO) and 10% OADC (oleic acid–albumin–dextrose–catalase; Becton Dickinson) until logarithmic growth was reached. Each culture was mixed with a sufficient volume of sterile supplemented Middlebrook 7H9 broth to achieve a turbidity equivalent to that of McFarland's N° 1 standard. To obtain the test inoculum, this suspension was further diluted 1:50 v/v with the same culture medium to approximately

$6x10^6$ colony-forming units (CFU/ml) immediately before use. The crude extract and

compounds were dissolved in 100% dimethyl sulfoxide (DMSO, Sigma), then diluted in a

fresh supplemented Middlebrook 7H9 broth. These samples as well as INH were diluted to

their final concentrations ranging from 0.31-78.12 µg/ml for the crude extract and fractions,

0.16-39.06 µg/ml for compounds **1** and **2**. The final concentration of DMSO in all assays was

2.5% or less, which is nontoxic for mycobacteria. The samples were assayed twice in

duplicate. All tests were carried out in sterile flat-bottomed 96-well microplates. Sterile

double-distilled water (100 µl) was poured into the wells on the outer perimeters of the

microplates, and 100 µl of Middlebrook 7H9 broth supplemented with OADC was added to

the remaining (test) wells. Each microplate was incubated for 5 days at 37°C in a 5% CO_2

atmosphere (in a sealed plastic CO_2-permeable bag). After 5 days of incubation, 32 µl of a

mixture of freshly prepared Alamar Blue solution (Sigma) and 20% sterile Tween-80 (Sigma)

1:1 v/v) were added to one growth-control well. The microplates were incubated again at

37°C for 24 h. If a color shift from blue to pink was observed in the growth-control sample,

32 µl of Alamar Blue solution was then added to each of the remaining wells, and the

microplate was further incubated for 24 h. A well defined pink color was interpreted as

positive bacterial growth, whereas a blue color indicated an absence of growth. The MIC

corresponded to the greatest dilution of sample extract in which the color shift from blue to

pink was not observed.

2.9. Determination of Mycobactericidal Effect (MMC)

Samples with detected MIC values with MABA (Collins and Franzblau 1997,

Jimenez-Arellanes et al. 2003) were assayed for their mycobactericidal effect as follows. Two

six-well rows of a microplate were prepared with fresh Middlebrook 7H9 culture medium.

Two-fold dilution series of the experimental sample and inoculum were set up as previously

described, but only one six-well row was used to confirm the MIC value with Alamar Blue.

Immediately thereafter, 5 µl of the undeveloped mycobacterial suspensions were transferred from the former to a new microplate that contained 195 µl of fresh culture medium per well. Three wells were inoculated with 100 µl of fresh inoculum as for MABA and three more wells were incubated with 200 µl of culture medium only (as negative controls). The microplates were incubated and developed with Alamar Blue as for MABA. The minimal bactericidal concentration (MMC) corresponded to the minimum sample concentration that did not cause a color shift in cultures re-incubated in fresh medium.

2.10. Preparation of discs

Whatmann filter paper (N° 1) discs of 6 mm diameter were impregnated with 10 µl of the crude extract solution at 10 mg/ml (100 µg/disc), fractions and isolated compounds at 4 mg/ml (40 µg/disc) prepared using DMSO. The discs were evaporated at 37°C for 24 hours. The RA discs and SA were prepared as described above using the appropriate concentrations to obtain discs containing 40 µg and 100 µg of drug respectively. Two discs were prepared for each sample.

2.11. Diffusion test

The antimicrobial diffusion test was carried out as described by Jorgensen et al. (1999) using a cell suspension of about 1.5 10^6 CFU/ml obtained from a McFarland turbidity standard N° 0.5. The suspension was standardized by adjusting the optical density to 0.1 at 600 nm (SHIMADZU UV-120-01 spectrophotometer) (Kuete et al., 2007a, b). 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 were 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 30°C for 48 hours (*M. audouinii* and *T. rubrum*) or 37°C for 24 hours (other organisms). Antimicrobial activity was evaluated by measuring the

diameter of the inhibition zone (IZ) around the disc. The assay was repeated twice in duplicate and results were recorded as mean ± SD of the duplicated experiment.

2.12. MIC and MMC determinations

The MICs of the crude extract, fractions, compounds **1** and **2** and reference antibiotics (RA) (gentamicin for bacteria and nystatin for fungi) were determined as follows; the test sample was initially dissolved in dimethylsulfoxide (DMSO). The solution obtained was then added to MHB to give a final concentration of 78.12 µg/ml. This was serially diluted two fold to obtain concentration ranges of 0.31 to 78.12 µg/ml. 100 µl of each concentration was added in a well (96- wells microplate) containing 95 µl of MHB and 5 µl of inoculum (standardized at $1.5 \ 10^6$ CFU/ml by adjusting the optical density to 0.1 at 600 nm SHIMADZU UV-120-01 spectrophotometer) (Kuete et al. 2007a, b). The final concentration of DMSO in the well was less than 1% (preliminary analyses with 1% (v/v) DMSO do not affect the growth of the test organisms). The negative control well consisted of 195µl of MHB and 5 µl of the standard inoculum (Kuete et al. 2007a, b). The plates were covered with a sterile plate sealer, then agitated to mix the contents of the wells using a plate shaker and incubated at 30°C for 48 hours (*M. audouinii* and *T. rubrum*) or 37°C for 24 hours (other organisms). The assay was repeated trice. The MIC of samples was detected following addition (40 µl) of 0.2 mg/ml *p*-iodonitrotetrazolium chloride and incubated at 37°C for 30 min (Kuete et al. 2008). Viable bacteria reduced the yellow dye to pink. MIC was defined as the lowest sample concentration that prevented this change and exhibited complete inhibition of bacterial growth.

For the determination of MMC, a portion of liquid (5 µl) from each well that showed no change in color was plated on MHA and incubated at 30°C for 48 hours (*M. audouinii* and *T. rubrum*) or 37°C for 24 hours (other organisms). The lowest concentration that yielded no growth after this sub-culturing was taken as the MMC (Kuete et al. 2007a, b).

3. Results and discussions

The Bio-assay guided fractionation of TSB led to the isolation of three main compounds, 3,4'-di-*O*-methylellagic acid 3'-*O*-β-D-xylopyranoside (**1**), 4'-*O*-galloy-3,3'-di-*O*-methylellagic acid 4-*O*-β-D-xylopyranoside (**2**) (Tabopda et al., 2006) and ellagic acid (**3**) (Grzegorz and Jaromir, 1996). These compounds together with 3,3'-di-*O*-methylellagic acid, 3,3'-di-*O*-methyl ellagic acid 4-*O*-β-D-xylopyranoside were previously isolated from *T. superba* (Tabopda et al. 2006). In the present report, we have evaluated the antmycobacterial, antibacterial and antifungal activities of *T. superba* extract, fractions and compounds **1** and **2** obtained following a bio-assay guided process. The results are recorded in Tables 1 to 3.

The results of the antimycobacterial assays (Table 2), showed that the crude extract, fraction TSB5-7 and compound **1** were able to prevent the growth of all the studied mycobacteria in the tested concentration range. The lowest MIC value (39.06 µg/ml) for this extract was recorded on both *M. smegmatis* and *M. tuberculosis* MTCS2. The corresponding values were 19.53 µg/ml for fractions (TSB6 on *M. smegmatis*) and 4.88 µg/ml for compounds (compound **1** on three of the four studied mycobacterial species). The MICs recorded with compound **1** (19.53 µg/ml) and **2** (39.06 µg/ml) on *M. tuberculosis* MTCS1 were lower than that of INH (>39.06 µg/ml). The obtained data highlighted the significant antimycobacterial potency of the two compounds as well as that of the crude extract, fractions TSB6 and TSB7. Results of the MMC determination (Table 3) showed detectable values for fraction TSB7 and compound **1.** A keen look of the results in Tables 2 and 3 shows that the recorded MMC values were not more than fourfold their corresponding MICs. This suggests that bactericidal effect of studied samples could be expected (Mims et al. 1993). The use of *M. smegmatis* in this assay was a preliminary step to select the concentration range to be tested on *M. tuberculosis* species. The results as obtained validated the necessity of such experiment. However, it is well known that the sensitivity of *M. smegmatis* is closer to that of

M. tuberculosis and that this non pathogenic mycobacterial species can be used in selecting samples for *M. tuberculosis* studies (Newton et al. 2002).

Tables 1 to 3 also summarize the results of the antimicrobial assays against fungi, Gram-positive and negative bacteria. Results of Table1 demonstrates that the crude extract from *T. superba* (TSB), fractions TSB6-7 as well as the two tested compounds exhibited microbial growth inhibition on all the tested organisms at the tested concentrations. The IZ obtained ranged from 13 to 19.5 mm for TSB, from10-19 mm and 14-20.5 mm respectively for TSB6 and TSB7. Compounds **1** and **2** showed IZ ranging from 12-21.5 mm and 9-19 mm respectively. Other fractions were selectively active. Their inhibition effects were noted on 9/11 (81.8%) tested microbial species for TSB4 and TSB5, 7/11 (74.3%) for TSB3, 6/11 (55.5%) for TSB2 and 4/11 (36.4%) for TSB1. The highest IZ were obtained against *M. audouinii* for TSB, and *S. aureus* for TSB8, compounds **1** and **2**.

The results of MIC determinations (Table2) indicate values ranging from 19.53 to 78.12 µg/ml for the TSB and compound **2** on 90.9% (10/11) of the tested organisms. Within the tested interval (0.31-78.12 µg/ml), compounds **1** as well as fractions TSB 6 and 7 showed evident MIC values on the entire set of the tested microbial strains. Against the 11 tested organisms, detectable MIC values were recorded on 4 (36.4%) for TSB2 and TSB5, 3 (27.3%) for TSB4, 2 (18.2%) for TSB3 and 1 (9.1%) for TSB1. The lowest MIC value for the crude extract (19.53 µg/ml) was recorded against *S. aureus*, *P. aeruginosa and M. audouinii.* This lowest value was also noted with fractions 6 and 7 on all tested fungi, and on most of the tested bacterial species. The corresponding value for the tested compounds (9.76 µg/ml) was obtained with compound **1** against *S. aureus* and *M. audouinii*. The reference antibiotics exhibited MIC values ranging from 4.88-39.06 µg/ml. The inhibition potential of the crude extract, fractions 6 and 7 as well as that of compounds **1** and **2** can be considered significantly important when regarding the antibacterial and antifungal activities of the RA. This can be

highlighted by the MIC value obtained for compound **1** on *M. audouinii* (activity two fold

greater than that of nystatin). Apart from the activity exhibited by compound **2** (MIC: 39.06

µg/ml) on *T. rubrum*, the preceding samples showed equal or greater antifungal effects than

nystatin on the four tested fungi. The results of the MMC determinations (Table 3) indicated

noticeable values for TSB on 36.4% (4/11) of the tested organisms. This assay also

confirmed the interesting activity of fractions 6, 7, compounds **1** and **2**. Detectable MMC

values were recorded against 90.1% (10/11) of the tested organisms for fraction TSB7,

compounds **1** and **2** and against 63.6% (7/11) for fraction TSB6. These data suggest that cidal

effect of the tested samples could be expected (Kuete et al. 2007a, b, c, 2008). Furthermore,

the activities of the crude extract, fractions 6 and 7 as well as that of the two tested

compounds **1** and **2** could be considered as very important since the tested organisms were

resistant to commonly used antibiotics. Compounds **1** and **2** appeared to be the main active

principles when considering the microorganisms tested in the present study.

The results from this study are in accordance with previous biological reports on the

genus *Terminalia*. The antimicrobial activity has been demonstrated for a number of

Termilia species such as *T. catappa* (Kloucek et al. 2005), *T. sericea* (Steenkamp et al. 2007),

T. glaucescens (Magassouba et al. 2007) etc. *T. chebula* ripe seeds extract was found to be

active against *Staphylocococcus aureus* (Bonjar 2004). Eldeen et al. (2006) demonstrated the

antibacterial activity (against both Gram-positive and Gram-negative bacteria) of anolignan B

isolated from *T. sericea*. Some of the bioactive compounds from *Terminalia* species include

23-galloylarjunolic acid and its β-glucopyranosyl ester, terminolic acid, arjunic acid,

arjungenin, arjunglucoside, sericic acid and sericoside isolated from *T. macroptera* (Conrad

et al., 1998). The present study also identified compounds **1** and **2** as other active principles

of the genus *Terminalia*. Antimicrobial pentacyclic triterpenoids from the stem bark were also

identified from this plant (Tabopda et al. 2009). Also, compounds **3** (ellagic acid) was

reported for its antimicrobial activities on *S. aureus, S. epidermidis, Micrococcus luteus, E. coli, B. subtilis, C. albicans* (Cowan 1999; Akiyama et al. 2001; Thiem and Goslinska, 2004). However, this compound was isolated in a limited amount in *T. superba* and could not be tested in this study. Ellagic acid like-compounds have been found to complex proteins (Scalbert 1991, Haslam 1996; Stern et al. 1996), inactivating microbial adhesions, enzymes, cell envelope transport proteins, etc.

The overall results provide promising baseline information for the potential use of the crude extracts from the stem bark of *Terminalia superba,* fractions 6 and 7 as well as the two tested isolated compounds in the treatment of tuberculosis, and other bacterial and fungal infections. However, this will further be confirmed by pharmacological and toxicological studies currently going on in our laboratory.

Acknowledgement

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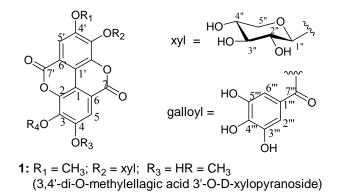   (Ed.), ASM Press, Washington D.C., pp. 1526-1643.

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1: R_1 = CH_3; R_2 = xyl; R_3 = HR = CH_3
(3,4'-di-O-methylellagic acid 3'-O-D-xylopyranoside)

2: R_1 = xyl; R_2 = CH_3; R_3 = galloyl; R_4=CH3
(4'-O-galloy-3,3'-di-O-methylellagic acid 4-O- -D-xylopyranoside)

3: R_1 = H; R_2 = H; R_3 = H; R_4= H
(Ellagic acid)

Figure1: chemical structures of compounds isolated from *T. superba*

Table1

Antimicrobial activity[a] of the crude extracts, fractions, compounds isolated from *T. superba* and reference antibiotics determined by the disc diffusion test

Tested organisms	Tested samples[b]												
	Crude extract	Fractions							Compounds		Antibiotics	
	TSB	TSB1	TSB2	TSB3	TSB4	TSB5	TSB6	TSB7	1	2	RA	SA
Bacteria												
Staphylococcus aureus	19.0±1.0	11.5±0.0	11.0±0.0	10.0±0.0	12.5±1.0	12.5±0.5	19.0±0.0	20.5±0.5	21.0±0.0	19.0±1.0	22.5±1.5	13.0±0.0
Escherichia coli	15.0±0.0	8.0±0.0	8.0±0.0	10.0±0.0	12.0±1.0	10.5±0.5	14.5±0.5	14.5±0.5	13.0±0.0	13.0±0.5	24.0±2.0	12.5±1.0
Shigella dysenteriae	17.5±1.5	-	-	-	10.5±0.0	13.0±0.0	15.5±0.5	16.0±0.0	16.0±0.0	16.0±0.5	21.5±0.5	14.5±1.0
Klebsiella pneumoniae	16.0±0.0	-	-	-	-	-	10.0±0.0	16.0±0.0	14.0±0.0	14.0±0.0	13.0±0.0	8.5±0.0
Pseudomonas aeruginosa	18.0±1.0	-	-	8.0±0.0	10.0±0.0	8.0±0.0	17.5±0.5	18.0±0.0	17.5±0.5	12.0±0.0	20.5±1.5	10.0±0.0
Salmonella typhi	15.0±0.0	8.0±0.0	12.5±1.0	10.0±0.0	8.5±0.0	-	16.0±0.0	16.0±0.0	18.5±0.5	16.5±0.5	18.0±0.0	14.5±0.5
Citrobacter freundii	13.0±0.0	-	-	-	-	8.0±0.0	12.5±0.5	14.0±1.0	12.0±0.0	9.0±0.0	21.5±1.0	11.0±0.0
Fungi												
Candida albicans	17.5±0.0	-	11.0±0.0	10.5±0.5	11.0±1.0	12.0±1.0	17.5±0.0	17.5±0.0	19.0±0.0	18.0±0.0	18.0±0.0	nt
Candida glabrata	14.0±0.0	-	-	-	8.0±0.0	8.0±1.0	15.5±0.5	17.0±0.0	15.5±0.5	16.0±1.0	17.5±0.0	nt
Microsporum audouinii	19.5±0.5	-	12.0±0.0	12.5±0.0	8.0±0.0	8.5±0.5	19.0±0.0	20.0±1.0	21.5±0.0	18.0±1.0	19.0±0.0	nt
Trichophyton rubrum	17.5±0.5	8.0±0.0	8.0±0.0	12.0±0.0	12.5±0.5	10.0±0.0	16.0±1.0	18.5±0.5	16.0±0.0	15.5±0.0	20.0±0.0	nt

[a]Antimicrobial activity: crude extract was tested at 100 µg/disc while fractions, compounds and RA at 40 µg/disc and SA at 100 µg/disc

[b]The Tested samples were crude extract from the stem bark of *T. superba* (TSB), fractions 1-7 (TSB1-7), **1**: 3,4'-di-*O*-methylellagic acid 3'-*O*-β-D-xylopyranoside **2***: 4'-*O*-galloy-3,3'-di-*O*-methylellagic acid 4-*O*-β-D-xylopyranoside, RA or Reference antibiotics (Gentamycin for bacteria, Nystatin for yeasts), SA: specific antibiotics (Methicillin for *Staphylococcus aureus*, amoxiccilin for *Escherichia coli* LMP701,*Shigella dysenteriae*, Ampicillin for *Klebsiella pneumoniae* , Carbenicillin for *Pseudomonas aeruginosa* , Chloramphenicol for *Salmonella typhi* and *Citrobacter freundii.*

(-): Not active;

(nt): not tested

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Table2

Minimum inhibition concentration (µg/ml) of the crude extracts, fractions, compounds isolated from *T. superba* and reference antibiotics

Tested organisms	Tested samples[b]												
	Crude extract	Fractions							Compounds		Antibiotics	
	TSB	TSB1	TSB2	TSB3	TSB4	TSB5	TSB6	TSB7	1	2	RA	SA
Mycobacteria												
Mycobcaterium smegmatis	39.06	78.12	>78.12	>78.12	78.12	78.12	19.53	39.06	4.88	19.53	0.61	nt
Mycobcaterium tuberculosis H37Rv (ATCC 27294)	78.12	78.12	>78.12	>78.12	78.12	78.12	39.06	39.06	4.88	9.76	0.31	nt
Mycobcaterium tuberculosis MTCS1	78.12	>78.12	>78.12	>78.12	>78.12	78.12	39.06	39.06	19.53	39.06	>39.06	nt
Mycobcaterium tuberculosis MTCS2	39.06	78.12	>78.12	>78.12	>78.12	78.12	39.06	39.06	4.88	9.76	0.31	nt
Bacteria												nt
Staphylococcus aureus	19.53	78.12	78.12	>78.12	78.12	78.12	19.53	19.53	9.76	39.06	9.76	39.06
Escherichia coli	78.12	>78.12	>78.12	>78.12	78.12	>78.12	39.06	39.06	39.06	39.06	4.88	>39.06
Shigella dysenteriae	39.06	-	-	-	>78.12	78.12	19.53	19.53	19.53	19.53	9.76	39.06
Klebsiella pneumoniae	78.12	-	-	-	-	-	78.12	19.53	39.06	39.06	39.06	>39.06
Pseudomonas aeruginosa	19.53	-	-	>78.12	>78.12	>78.12	19.53	19.53	19.53	39.06	9.76	>39.06
Salmonella typhi	78.12	>78.12	78.12	>78.12	>78.12	-	19.53	19.53	19.53	19.53	19.53	39.06
Citrobacter freundii	>78.12	-	-	-	-	78.12	39.06	78.12	78.12	>78.12	9.76	>39.06
Fungi												
Candida albicans	39.06	-	78.12	>78.12	>78.12	78.12	19.53	19.53	19.53	19.53	19.53	nt
Candida glabrata	78.12	-	-	-	>78.12	>78.12	19.53	19.53	19.53	19.53	19.53	nt
Microsporum audouinii	19.53	-	78.12	78.12	>78.12	>78.12	19.53	19.53	9.76	19.53	19.53	nt
Trichophyton rubrum	39.06	>78.12	>78.12	78.12	78.12	>78.12	19.53	19.53	19.53	39.06	9.76	nt

[a]The tested microorganisms were *S. aureus*: *Staphylococcus aureus*, *E. coli*: *Escherichia coli*, *S. dysenteriae*: *Shigella dysenteriae*, *K. pneumoniae*: *Klebsiella pneumoniae*, *P. aeruginosa*: *Pseudomonas aeruginosa*, *S. typhi*: *Salmonella typhi*, *C. freundii*: *Citrobacter freundii*, *C. albicans*: *Candida albicans*, *C. glabrata*: *Candida glabrata*, *M. audouinii*: *Microsporum audouinii*, *T. rubrum*: *Trichophyton rubrum*;

[b]The Tested samples were crude extract from the stem bark of *T. superba* (TSB), fractions 1-7 (TSB1-7), **1**: 3,4'-di-*O*-methylellagic acid 3'-*O*-β-D-xylopyranoside **2**: 4'-*O*-galloy-3,3'-di-*O*-methylellagic acid 4-*O*-β-D-xylopyranoside, RA or Reference antibiotics (Gentamycin for bacteria, Nystatin for yeasts, isoniasid for *M. tuberculosis*); SA: specific antibiotics (Methicillin for *Staphylococcus aureus*, amoxiccilin for *Escherichia coli* LMP701,*Shigella dysenteriae*, Ampicillin for *Klebsiella pneumoniae* , Carbenicillin for *Pseudomonas aeruginosa* , Chloramphenicol for *Salmonella typhi* and *Citrobacter freundii*.

 (-): Not determined as the sample was not active following the diffusion test

(nt): not tested

Table3

Minimum microbicidal concentration (µg/ml) of the crude extracts, fractions, compounds isolated from *T. superba* and reference antibiotics

Tested organisms[b]	Tested samples[b]												
	Crude extract	Fractions								Compounds		Antibiotics	
	TSB	TSB1	TSB2	TSB3	TSB4	TSB5	TSB6	TSB7	1	2	RA	SA
Mycobcaterium smegmatis	78.12	>78.12	nd	nd	>78.12	>78.12	39.06	78.12	9.76	>39.06	2.44	nt
Mycobcaterium tuberculosis H37Rv (ATCC 27294)	>78.12	>78.12	nd	nd	>78.12	>78.12	78.12	78.12	9.76	19.53	0.61	nt
Mycobcaterium tuberculosis MTCS1	>78.12	nd	nd	nd	nd	>78.12	>78.12	78.12	39.06	>39.06	nd	nt
Mycobcaterium tuberculosis MTCS2	78.12	>78.12	nd	nd	nd	>78.12	78.12	78.12	9.76	39.06	0.61	nt
Bacteria												
Staphylococcus aureus	39.06	>78.12	>78.12	nd	>78.12	>78.12	>78.12	78.12	39.06	78.12	19.53	>39.06
Escherichia coli	>78.12	nd	nd	nd	>78.12	nd	>78.12	78.12	78.12	78.12	9.76	nt
Shigella dysenteriae	78.12	-	-	-	nd	>78.12	39.06	39.06	39.06	39.06	19.53	78.12
Klebsiella pneumoniae	>78.12	-	-	-	-	-	>78.12	78.12	78.12	78.12	78.12	nt
Pseudomonas aeruginosa	78.12	-	-	nd	nd	nd	78.12	39.06	39.06	78.12	19.53	nt
Salmonella typhi	>78.12	nd	>78.12	nd	nd	-	39.06	39.06	39.06	39.06	39.06	>39.06
Citrobacter freundii	nd	-	-	-	-	>78.12	78.12	>78.12	>78.12	nd	19.53	nt
Fungi												nt
Candida albicans	>78.12	-	>78.12	nd	nd	>78.12	39.06	39.06	39.06	39.06	39.06	nt
Candida glabrata	>78.12	-	-	-	nd	nd	>78.12	78.12	78.12	78.12	39.06	nt
Microsporum audouinii	39.06	-	>78.12	>78.12	nd	nd	39.06	39.06	19.53	39.06	39.06	nt
Trichophyton rubrum	>78.12	nd	nd	>78.12	>78.12	nd	78.12	39.06	39.06	78.12	19.53	nt

[a]The tested microorganisms were *S. aureus*: *Staphylococcus aureus*, *E. coli: Escherichia coli*, *S. dysenteriae*: *Shigella dysenteriae*, *K. pneumoniae*: *Klebsiella pneumoniae*, *P. aeruginosa*: *Pseudomonas aeruginosa*, *S. typhi*: *Salmonella typhi*, *C. freundii*: *Citrobacter freundii*, *C. albicans*: *Candida albicans*, *C. glabrata*: *Candida glabrata*, *M. audouinii*: *Microsporum audouinii*, *T. rubrum*: *Trichophyton rubrum*;

[b]The Tested samples were crude extract from the stem bark of *T. superba* (TSB), fractions 1-7 (TSB1-7), **1**: 3,4'-di-*O*-methylellagic acid 3'-*O*-β-D-xylopyranoside **2***:* 4'-*O*-galloy-3,3'-di-*O*-methylellagic acid 4-*O*-β-D-xylopyranoside, RA or Reference antibiotics (Gentamycin for bacteria, Nystatin for yeasts, isoniasid for *M. tuberculosis*), SA: specific antibiotics (Methicillin for *Staphylococcus aureus*, amoxiccilin for *Escherichia coli* LMP701,*Shigella dysenteriae*, Ampicillin for *Klebsiella pneumoniae* , Carbenicillin for *Pseudomonas aeruginosa* , Chloramphenicol for *Salmonella typhi* and *Citrobacter freundii*.

(-): Not tested as the MIC was not determined; (nd): not determined because the MMC>78.12 µg/ml (nt): not tested