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Terminalia laxiflora and *Terminalia brownii contain a broad spectrum of antimycobacterial compounds including ellagitannins, ellagic acid derivatives, triterpenes, fatty acids and fatty alcohols*

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Abstract

***Ethnopharmacological relevance**:*

Terminalia laxiflora Engl. & Diels, (Sudanese Arabic name: Darout الدروت) and *Terminalia brownii* Fresen (Sudanese Arabic name: Alshaf ألشاف) (Combretaceae) are used in Sudanese traditional folk medicine and in other African countries for treatment of infectious diseases, TB and its symptoms, such as cough, bronchitis and chest pain.

***Aim of study**:*

Because of the frequent use of *T. laxiflora* and *T. brownii* in African traditional medicine and due to the absence of studies regarding their antimycobacterial potential there was a need to screen extracts of *T. laxiflora* and *T. brownii* for their growth inhibitory potential and to study the chemical composition and compounds in growth inhibitory extracts.

Materials and methods:

The plant species were collected in Sudan (Blue Nile Forest, Ed Damazin Forestry areas) and selected according to their uses in traditional medicine for the treatment of bacterial infections, including TB. Eighty extracts and fractions of the stem bark, stem wood, roots, leaves and fruits of *T. laxiflora* and *T. brownii* and nine pure compounds present in the active extracts were screened against *Mycobacterium smegmatis* ATCC 14468 using agar diffusion and microplate dilution methods. Inhibition zones and MIC values were estimated and compared to rifampicin. HPLC-UV/DAD, GC/MS and UHPLC/Q-TOF MS were employed to identify the compounds in the growth inhibitory extracts.

Results:

The roots of *T. laxiflora* and *T. brownii* gave the best antimycobacterial effects (IZ 22-27 mm) against *Mycobacterium smegmatis*. The lowest MIC of 625µg/ml was observed for an acetone extract of the root of *T. laxiflora* followed by methanol and ethyl acetate extracts, both giving MIC values of 1250 µg/ml. Sephadex LH-20 column chromatography purification of *T. brownii* roots resulted in low MIC values of 62.5 µg/ml and 125 µg/ml for acetone and ethanol fractions, respectively, compared to 5000 µg/ml for the crude methanol extract. Methyl (*S*)-flavogallonate is suggested to be the main active compound in the Sephadex LH- 20 acetone fraction, while ellagic acid xyloside and methyl ellagic acid xyloside are suggested to give good antimycobacterial activity in the Sephadex LH-20 ethanol fraction. RP-18 TLC purifications of an ethyl acetate extract of *T. laxiflora* roots resulted in the enrichment of punicalagin in one of the fractions (Fr5). This fraction gave a five times smaller MIC (500 µg/ml) than the crude ethyl acetate extract (2500 µg/ml) and this improved activity is suggested to be mostly due to punicalagin. 1,18-octadec-9-ene-dioate, stigmast-4-en-3-one, 5α-stigmastan-3,6-dione, triacontanol, sitostenone and β-sitosterol were found in antimycobacterial hexane extracts of the stem bark of both studied species. Of these compounds, 1,18-octadec-9-ene-dioate, stigmast-4-en-3-one, 5α-stigmastan-3,6-dione, triacontanol, sitostenone have not been previously identified in *T. brownii* and *T. laxiflora*. Moreover, both plant species contained friedelin, betulinic acid, β-amyrine and two unknown oleanane-type triterpenoids. Of the listed compounds, friedelin, triacontanol and sitostenone gave a MIC of 250 µg/ml against *M. smegmatis*, whereas stigmasterol and β-sitosterol gave MIC values of 500 µg/ml.

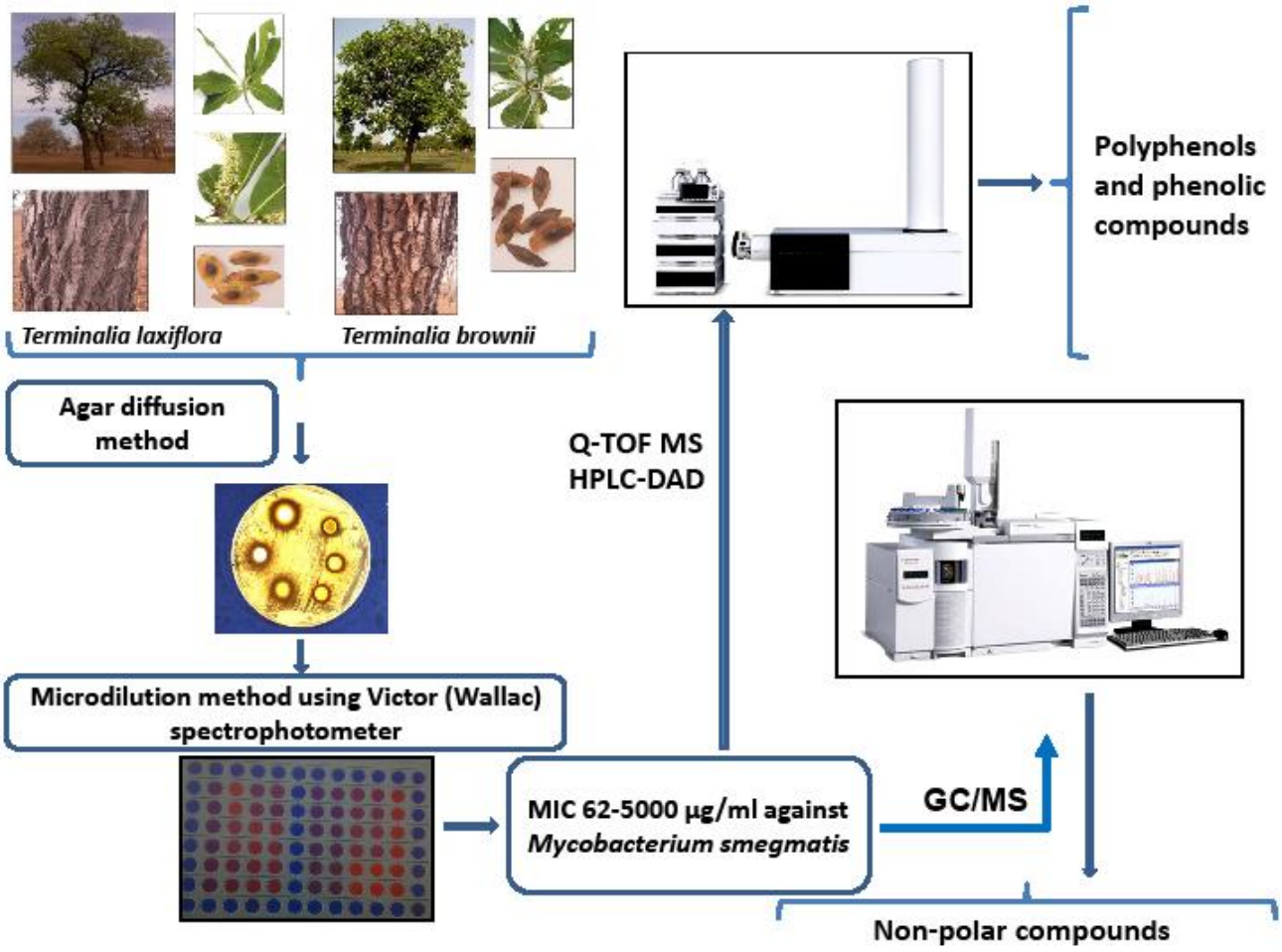
Conclusions*:*

Our results show that *T. laxiflora* and *T. brownii* contain antimycobacterial compounds of diverse polarities and support the traditional uses of various parts of *T. laxiflora* and *T.brownii* as

decoctions for treatment of tuberculosis. Further investigations are warranted to explore additional (new) antimycobacterial compounds in the active extracts of *T. laxiflora* and *T. brownii*.

Abbreviations

Graphical abstract



HPLC-DAD, High performance liquid chromatography with diode-array detection; UHPLC/QTOF-MS, Ultra-high performance liquid chromatography coupled to quadrupole time of flight mass spectrometry; $[M-H]^-$, deprotonated molecular ion ; Rt, retention time; R_f, retardation factor; $UV_\lambda max.$, Ultraviolet maximum absorption; Ppm, parts per million mass error ; ET, Ellagitannin; GC/MS, Gas chromatography coupled to mass spectrometry; M^+, Molecular ion ; m/z, Mass-to-charge ratio; TMS, Trimethylsilyl; BSTFA, N,O-bis (trimethylsilyl)-trifluoroacetamide; TMCS, Trimethylchlorosilane; TLC, Thin layer chromatography; MIC, Minimum inhibitory concentration; IZ, Diameter of inhibition zone in mm ; IC, Inhibitory concentration ; SAR, Structure-activity relationship; TB, Tuberculosis; MDR, Multi drug resistant; XDR, Extended drug resistant; MRSA, Multi drug resistant *Staphylococcus aureus;* VRE, Vancomycin resistant Enterococci; TMP, Traditional medicinal practitioner; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; HHDP, Hexahydroxydiphenoyl unit ; DHHDP, Dehydroxyhexahydroxydiphenoyl unit

Keywords: *Mycobacterium smegmatis*, *Terminalia laxiflora*, *Terminalia brownii*, ellagitannins, triterpenoids, fatty compounds

Chemical compounds identified and studied in this article:

Ellagic acid (PubChem CID: 5281855), Punicalagin (PubChem CID: 16148440), Corilagin (PubChem CID: 73568), Friedelin (PubChem CID: 91472), Triacontanol (PubChem CID: 68972), Sitostenone (PubChem CID: 241573), β-Sitosterol (PubChem CID: 222284)

1. Introduction

Tuberculosis (TB), is caused by the globally leading bacterial killer, *Mycobacterium tuberculosis*, and is becoming increasingly common worldwide due to international travel (WHO, 2006; Willcox et al., 2004). Presently, TB is a major health hazard due to multidrug-resistant (MDR) and extensively-drug resistant (XDR) forms of *M. tuberculosis* which emerge specifically in poor societies due to inaccurate medication and poor living standards (WHO, 2013a; Zhao et al., 2014). Among children, pregnant women and people suffering from immunodeficiency, the mortality rate due to TB is especially high (WHO, 2010). In 2012, TB resulted in 1.3 million deaths and 8.6 million new TB cases (Zhao et al., 2014; WHO, 2010). It is estimated that about 3.5% of the new cases and 20.5% of earlier treated have MDR-TB (WHO, 2014). Besides, an estimated 9.6% of the MDR-TB cases have appeared to be of the XDR-TB form (WHO, 2013b). One of the limitations to the eradication of TB is its ability to persist in the human lungs in a dormant state, and this state is especially resistant to anti-TB drugs and to the human immune system (Sala et al., 2014). Latent infection is the major pool of worldwide TB cases and treatment of latent TB is therefore an important goal to eradicate the disease (Young et al., 2009; Abuhammad et al., 2012). Moreover, the six month long treatment regimens of TB, using rifampin, isoniazid, ethambutol and pyrazinamide, often gives severe side effects and is difficult to complete in impoverished areas with poor public health care systems. Treating resistant TB requires even longer treatment periods and more toxic therapies (Wivagg et al., 2014).

The current number of new anti-TB candidates in the drug pipeline for combating TB is insufficient (Marrakchi et al., 2014; Zumla et al., 2013). Therefore, the need to find and develop new anti-TB drugs is urgent (Shilpi et al., 2015). In recent years there has been a renewed interest in the discovery of antimycobacterial agents from natural sources (Dashti et al., 2014; Santhosh and Suriyanarayanan, 2014; Shilpi et al., 2015).

Africa has a rich tradition on the uses of medicinal plants for the treatment of tuberculosis and its symptoms and this ethnobotanical information could be used as a guideline to find new scaffolds for anti-TB drugs from African plants. In Africa there occurs an estimated 30 species of *Terminalia* and only a fraction of these species have been studied for their antimycobacterial effects and active compounds, although most of them have some uses for treatment of TB or its symptoms. For example, antimycobacterial ellagic acid xyloside and punicalagin were found from *T. superba* (Kuete et al., 2010); acetone extracts of *T. sericea* inhibited the growth of *M. tuberculosis* H_{37} Ra (Green et al., 2010); crude extracts of the leaves of *T. glaucescens* were active against *M. tuberculosis* H_{37} Rv (Nvau et al., 2011); the pentacyclic triterpene, friedelin, from *T. avicennoides* gave good growth inhibitory effects against *M. bovis* (Mann et al., 2011).

Terminalia laxiflora (Engl. & Diels) and *T. brownii* (Fresen) (Comretaceae) are dry savanna woodland trees occurring commonly in the Sudano-Sahelian area of Africa (Foyet, 2013). Other additional geographical regions of occurrence for *T. brownii* include Nigeria, Congo, North Tanzania and Kenya (Mosango, 2013). *T. laxiflora* and *T. brownii* are customarily used by traditional medicinal practitioners (TMP) against cough, chest pain and fever, symptoms related to TB (Mohieldin et al., 2017; El Ghazali et al., 2003; Musa et al., 2011; El Ghazali et al., 1997; Salih personal communication, 2006, 2012 and 2014). In Sudan, traditional preparations of remedies from *T. brownii* and *T. laxiflora* include soaking fresh and/or dry plant material for one day in lukewarm tap water as well as boiling the plant material in water for a few minutes to prepare decoctions, or to infuse the plant material in freshly boiled water to produce a tea (Muddathir et al., 2013; El Ghazali et al., 2003; Salih personal communication, 2006, 2012 and 2014). Despite of the frequent and various uses of *T. brownii* and *T. laxiflora* for the treatment of bacterial infections in traditional medicine, there are few studies available on their antimicrobial activities (Opiyo et al., 2011; Mbwambo et al., 2007) and to the best of our knowledge no studies have been conducted previously on the antimycobacterial effects of extracts and compounds of these plants.

Some research has been done on the phytochemistry of *Terminalia laxiflora* and *T. brownii*. Medium polar to polar phenolic compounds, such as gallic acid, ellagic acid and its derivatives, gallotannins and the ellagitannins punicalagin, terchebulin, methyl-(*S*)-flavogallonate and its isomer have recently been characterized in the roots of both species and in the stem bark of *T. brownii* (Machumi et al., 2013; Yamauchi et al., 2016; Schrader et al., 2016; Salih et al., 2017). Moreover, terchebulin, flavogallonic acid dilactone and ellagic acid and its derivatives have been reported to occur in the stem wood of *T. laxiflora* (Ekong and Idemudia,1967; Muddathir et al., 2013). In addition, the sterols β-sitosterol and stigmasterol as well as the triterpenoids betulinic

acid, arjungenin, terminolic acid and monogynol A, the diterpenoid laxiflorin and terminalianone, a chromone, were reported from the stem bark of *Terminalia brownii* and *T. laxiflora* and the root bark of *Terminalia laxiflora* (Rashed et al., 2016; Machumi et al., 2013; Opiyo et al., 2010; Negishi, et al., 2011; Ekong and Idemudia, 1967).

This study describes the growth inhibitory effects of extracts of a wide range of polarities from the stem bark, stem wood, roots, leaves and fruits of *T. brownii* and *T. laxiflora* on *Mycobacterium smegmatis*. Sephadex LH-20 and RP-18 TLC fractions, enriched in ellagitannins and ellagic acid derivatives, were also used in the screenings in order to investigate how these chromatographic purifications affect the capacity of the fractions to inhibit the growth of *M. smegmatis*. The MIC values of some pure compounds occurring in the extracts were investigated in order to compare their activities to the extracts. UHPLC/Q-TOF MS, HPLC-DAD and GC/MS results on the molecular masses of ellagitannins, ellagic acid derivatives, triterpenes, sterols, fatty acids and fatty alcohol derivatives are presented.

2. Material and methods

2.1. Plant material

Dried leaves, fruits, stem bark, stem wood and roots of *T. brownii* and *T. laxiflora*, collected in May-June 2006, in February-March 2012 and July-August 2014 in Ed Damazin Forestry areas, south-eastern Sudan, were used in this study. Mr. Ashraf Mohamed Ahmed Abd Alla (PhD) and Mr. Haytham Hashim Gibreel (PhD) at the Faculty of Forestry, University of Khartoum, Sudan and Mr. El Sheikh Abd alla Al Sheikh (PhD) at Soba Forest Research Center, Khartoum, Sudan confirmed the identity of the plants used in this study. Voucher specimen of *Terminalia laxiflora* (Plate 24. June, 2006) and *T. brownii* (Plate 23. June, 2006) are deposited in the Herbarium at University of Khartoum, Sudan. The collected plants were shade dried at + 28°C, separated manually to stem wood, stem bark, roots, leaves and fruits and ground to fine powder using a hammer mill (mesh). This powder was used for extraction, antimycobacterial testing and phytochemical analysis.

2.2. Extraction methods

2.2.1. Soxhlet extraction

Methanol was used in order to obtain crude extracts containing a broad class of compounds. 20 g of dried ground materials were extracted with 800 ml MeOH in a Soxhlet apparatus for 5 hours. The obtained extracts were cooled before transferring to a rotary evaporator (Heidolph VV2000), using a water bath not exceeding +40°C. The extracts were then freeze-dried in a lyophilizer for 1–2 days.

2.2.2. Sequential extraction and liquid partition

Sequential extraction and liquid partition was used to obtain extracts of various polarities in order to monitor compound classes responsible for antimycobacterial activity. In brief, for sequential extraction, 100 g of plant powder was defatted using hexane (1500 ml), whereafter the marc was extracted with dichloromethane or chloroform (1400 ml) followed by 80% methanol (800-1000 ml). For root bark, roots and leaves the marc was extracted with acetone (800-1000 ml) before using 80 % methanol. The 80 % methanol extraction was completed using a magnetic stirrer overnight at room temperature. The 80% methanolic extracts were fractionated with ethyl acetate (EtOAc) using liquid-liquid partition. Thus, the extractions and fractionations resulted in hexane, dichloromethane, chloroform, acetone, ethyl acetate and aqueous fractions. Ethyl acetate and aqueous fractions were concentrated to dryness using a rotary evaporator (Heidolph VV2000) at +40° C. The rest of the fractions were dried at room temperature in a fume cabinet. All extracts were further freeze-dried in a lyophilizer

2.2.3. Decoctions, macerations and cold methanol extractions

Decoctions were prepared to resemble as closely as possible similar preparations customarily used by traditional healers in Sudan. 500 ml of water was added to 20 g of the plant powder, mixed carefully and brought to the boil. The decoction was centrifuged at 2000 rpm for 10-15 minutes (Eppendorf AG centrifuge 5810 R, Germany) and filtered using filter paper (Schleicher & Schuell, ∅ 150 mm, Germany), whereafter it was freeze-dried for two days. Macerations were prepared from 10-20 g of plant powder soaked in 300-500 ml cold water overnight. Cold methanol extractions were prepared according to a procedure identical to the macerations, using the same extraction time and dry material to solvent ratio (w/v).

Extracts resulting from the various extraction procedures described above were dissolved in methanol (medium-polar to polar extracts) or hexane (very non-polar extracts) to a concentration of

50 mg/ml and were used for primary antimycobacterial screening using an agar disk diffusion method. The yields resulting from each method of extraction are given in Fig. S8 and S9.

2.3. Chromatography

2.3.1. Sephadex LH-20 chromatography of a Terminalia brownii root extract

A root methanolic Soxhlet extract of *Terminalia brownii* was subjected to Sephadex LH-20 (Pharmacia, Uppsala) fractionation using size exclusion in order to separate tannins from non-tannin phenols in the extract (Hagerman, 2002; Saleem et al., 2002). 200 mg dry root methanolic Soxhlet extract was dissolved in 10 ml of 80% (v/v) EtOH. The extract was centrifuged to avoid precipitation whereafter it was poured into a 50 ml volume plastic centrifuge tube (Eppendorf) containing 2.5 g Sephadex LH-20 (Uppsala, Pharmacia Biotech AB, Sweden). The Sephadex LH-20 and the extract in the tube were mixed carefully, centrifuged at 3000 rpm for 3 min, whereafter the supernatant was collected. Extractions using 8-10 ml 80% (v/v) EtOH and centrifugations were repeated several times until the resulting supernatant changed color from yellow to clear and showed an absorbance close to zero at 280 nm. The "tannin fraction" (ET enriched fraction or acetone fraction) was released from the Sephadex LH-20 using 2 × 15 ml 70% acetone. The acetone extract was filtrated using double filter paper (Whatman) into a 100 ml rotary evaporation flask to be concentrated to dryness using a rotary evaporator. The 80 % EtOH fraction (containing a higher percentage of ellagic acid derivatives) was also reduced to dryness using a rotary evaporator. Both fractions were dissolved in 5 ml H_2O before drying, using liquid nitrogen, whereafter the fractions were freeze-dried for two days. The freeze-dried fractions were dissolved in MeOH to make stock solutions of 1000 µg/ml for antimycobacterial testing and for phytochemical analysis (HPLC-DAD).

2.3.2. Preparative RP-18 TLC of a Terminalia laxiflora root extract

Preparative thin layer chromatography (TLC) was performed on glass-backed RP-18 F_{254s} TLC-plates (Merck, Darmstadt, Germany) to separate ellagitannin enriched fractions. 10 microliters of extracts and fractions (50 mg/ml) were applied equidistantly from each other and 1.5 cm from the bottom of the plate using a microcapillary pipette. 10 ml of freshly prepared methanol: water: orthophosphoric acid (50:50:1, v:v:v) was used as eluent and the development distance was 8-12 cm. Gallic acid, lutein, quercetin, corilagin and ellagic acid (Sigma-Aldrich) were used as

standard compounds. The developed plates were observed and photographed with a Camaq Reprostar 3 TLC Visualizer documentation system. The spots were marked at 366 nm (fluorescing) and 254 nm (quenching) wavelengths and the retardation factor (R_f values) were calculated (Fig. S1, S2 and S3). TLC spots were scraped from a large number of thin layer chromatography glass plates (30 plates approximately) and collected into centrifuge tubes (15-50ml). The silica powder containing the fractions/ compounds was extracted with methanol whereafter the tubes were centrifuged for 10 min using 5000 rpm (Mini Spin®plus, Eppendorf, Germany). The supernatants were carefully transferred to new test tubes and evaporated to dryness in an evaporator (Concentrator Plus, Eppendorf). The dried fractions were dissolved in methanol to a concentration of 1000 µg/ml stock solutions used for antimycobacterial testing and for phytochemical analysis (HPLC-DAD).

2.3.3. Qualitative DPPH-TLC bioautography method

A thin-layer chromatography bioautography method (Wang et al., 2012) for *in situ* qualitative determination of antioxidative effects of compounds and fractions of *T. brownii* and *T. laxiflora* was used. Thin-layer chromatography was performed on aluminium-backed RP-18 F_{254s} TLC plates (Merck, Darmstadt, Germany). 1 µl of extracts (50 mg/ml) were applied 1.5 cm from the bottom of the plate. Methanol: water: orthophosphoric acid (50:50:1,v:v:v) was used as eluent and development distance was 8-12 cm. The plates were left to dry for 30 min, wherafter they were photographed using Camaq Reprostar 3 TLC Visualizer documentation system at 366 and 254 nm. After documentation, the plates were sprayed evenly with 0.2 % (w/v) 2,2-Diphenyl-1-picrylhydrazyl reagent in methanol (DPPH, Sigma-Aldrich), left to dry and quickly photographed in visible light. Antioxidative fractions/compounds on the plate appear yellow against a purple background. Ellagic acid, gallic acid, luteolin, corilagin and naringenin (Sigma-Aldrich) were used as standard compounds (Fig. S3). The spayed plates were compared to their unsprayed replicates as well as with standard compounds and their R_f values in order to identify antioxidative compounds/fractions in extracts of *T. brownii* and *T. laxiflora*.

2.3.4. HPLC-UV/DAD method

A method described in Fyhrquist et al. (2014) and Salih et al. (2017) was used for the identification of polyphenols. An autosampler controlled by Agilent Chemstation software (Water Corp., Milford, USA) was used. A liquid chromatographic system was used consisting of a Waters 600 E pump and a controller coupled to a 991 PDA detector. Separations were made on a reversed

phase Hypersil Rp C_{18} column (length: 60 mm; ID: 2 mm). 10 µl of samples (2 mg/ml in 50% MeOH) were injected. Gradient elution was performed using two solvent systems: A) Aqueous 1.5% tetrahydrofuran + 0.25% orthophosphoric acid and B) 100 % MeOH. Flow rate was 2 ml/min. UV fingerprint chromatograms were constructed at 220, 270, 280, 320 and 360 nm. UV_{λ} absorption spectra of the compounds were recorded between 210 and 400 nm using Agilent Chemstation software. Phenolic compounds were compared to the computer compound library (Agilent Chemstation) and to the literature (Conrad et al., 2001; Pfundstein et al., 2010).

2.3.5. UHPLC/ Q-TOF MS method

UHPLC-DAD (Model 1200 Agilent Technologies)-JETSTREAM/QTOFMS (Model 6340 Agilent Technologies) equipped with a 2.1 × 60 mm, 1.7 µm C_{18} column (Agilent technologies) as described in Salih et al. (2017) was used for detecting the masses of polyphenolic compounds. Solvent A consisted of 1.5% tetrahydrofuran and 0.25% acetic acid in HPLC quality water and solvent B was 100% methanol. A gradient run was used as follows: from 0 to 1.5 min, B 0 %, from 1.5 to 3 min, 0 to 15% B, from 3 to 6 min, 10 to 30 % B, from 6 to 12 min, 30 to 50% B, from 12 to 20 min, 50 to 100% B, and from 20 to 22 min, 100 to 0% B. The qtof-mass spectra were acquired at the negative ion modes depending on the compounds, and a mass range from 100 to 2000 m/z was used. The ppm values describing the mass measurement error were calculated according to the below formula (Brenton and Godfrey, 2010):

Difference between an individual measurement and the calculated value, ΔMi (in ppm, parts per million mass error) = $(M_{measured} - M_{calculated}) \times 10^6 / M_{calculated}$

Where $M_{measured}$ stands for the measured mass in Q-TOF and $M_{calculated}$ stands for the calculated mass according to the molecular formula of the compound. Since negative mode of Q-TOF was used, the mass of the hydrogen atom (1.0078) was subtracted from all the calculated masses.

2.3.6. GC/MS analysis

2.3.6.1. Silylation

Trimethylchlorosilane [TMCA, GC grade] and N,O-bis (trimethylsilyl) trifluoroacetamide [BSTFA, GC grade] were purchased from Sigma (Buchs, Switzerland). According to silylation method by Münger et al. (2015), 10 mg of hexane extracts of the stem bark of *T. laxiflora* and *T. brownii* were dissolved in 2 ml dichloromethane (HPLC grade). 200µl of dissolved extracts were dried with nitrogen gas (PIERCE Modle 18780 Reacti-Vap™) and the

residue was mixed with 100µl of pyridine and 100µl of silylation reagent **[**BSTFA 99 %+ TMCS 1%]. The analytes were silylated in closed vials incubated in an oven (Horo) at 60^{O} C for 30-40 min, after which the mixtures containing trimethyl silyl ethers (TMS) were dried again with nitrogen gas. To prepare the sample for the GC/MS analysis, the dried samples were dissolved in 200µl of heptane (chromasolv® for HPLC).

2.3.6.2. Gas chromatograph mass spectrometers (GC/MS)

The GC/MS HP6890 instrument consisted of an autosampler, a split/splitless injector, a column oven and a MS detector. The capillary column used was a RTX^{TM}-5 fused-silica column [crossbond 5% diphenyl and 95% dimethyl polysiloxane phase; 60 m, 0.32 mm id, 0.1 mm film, Restek Corp., Bellefonte, PA, USA]. The injector temperature was set at 275^{O}C. The GC oven temperature was initially adjusted at 150^{O}C held for 1 min then set to 275^{O}C at a rate of 15 ^{O}C/min, and finally programmed to 310^{O}C at rate 5.00 ^{O}C/min and held for 10 min. The actual injected volume of the sample was 2.0 µl. Helium was used as the carrier gas and its pressure was adjusted at 21.50 psi. In the MS, the interface and ion source temperatures were 280^{O}C and 230^{O}C, and electron impact ionization was used. Full scan mass spectra (80-650 m/z) were collected.

2.3.6.3. GC/MS data analysis

The non-polar compounds were identified by comparing with mass spectra of standard compounds and to literature (AOCS libid library, Goad and Akihisa, 1997 and Assimopoulou and Papageorgiou, 2005) as well as Wiley, NIST and Scifinder libraries of reference compounds. The peak area (%) and M^{+} (the mass to charge for the molecular ion) of the compounds were reported.

2.4. Assays for measuring antimycobacterial activity

2.4.1. Agar disk diffusion

An agar disc diffusion assay according to Fyhrquist et al. (2014) was used for the primary antimycobacterial screening. *Mycobacterium smegmatis* ATCC 14468 was selected as test bacterium since it is known to have a similar drug sensitivity profile to *M. tuberculosis* and at the same time it is fast-growing and non-pathogenic (Newton et al., 2002). *M. smegmatis* was incubated on Löwenstein-Jensen agar slants (Becton-Dickinson & Company, USA) for five days at +37 °C. 200 µl bacterial culture containing 1.0×10^{8} CFU/ml was grown on Petri dishes containing 25 ml Middlebrook 7H10 agar (Difco) enriched with oleic acid, albumin, dextrose and catalase (OADC

supplement, Difco) as top layer and 25 ml Base agar (Difco) as base layer. 200 µl extracts (50mg/ml) and rifampicin (10mg/ml, Sigma-Aldrich) were applied on sterile filter paper disks (Ø = 12.7 mm, Schleicher and Schuell 2668). The solvent from the filter papers containing the extracts and antibiotics was left to dry. Methanol and hexane were used as negative controls. Prior to incubation the petri dishes were kept in +4 °C to facilitate diffusion of the extracts into the agar. The petri dishes were then incubated at +37 °C for five days. All extracts and antibiotics were tested in triplicate. The diameters of the zones of inhibition (IZ) were measured with a caliper under a petri dish magnifier and the mean of three replicate diameters ± SEM was calculated. Activity index of the plant extracts and fractions was measured in relation to rifampicin as in Fyhrquist et al. (2014) as follows:

AI (Activity index) = Inhibition zone of the plant extract/ Inhibition zone of rifampicin

2.4.2. Agar disk diffusion for MIC estimations

For some of the extracts MIC was difficult/impossible to determine using the microplate method due to extensive buildup of precipitation and in these cases the agar diffusion method described above was used. Two-fold dilutions from 5000 to 39.06 µg/ml for the plant extracts and from 1000 to 0.98 µg/ml for rifampicin were made in methanol or hexane. 200 µl of the dilutions were pipetted on filter paper disks (Ø = 12.7 mm, Schleicher and Schuell 2668) which were placed equidistantly on the petri dishes inoculated with *M. smegmatis* as described above. The petri dishes were incubated for five days in +37 °C, whereafter diameters of inhibition were measured as above. The approximate MIC was estimated as the lowest mean inhibitory concentration of triplicates giving a small, but visible inhibition zone around the filter paper disc.

2.4.3. Microplate broth dilution method

To determine the minimum inhibitory concentration (MIC) and the percentage inhibition of bacterial growth (inhibitory concentration IC) of those extracts giving the most prospective growth inhibitory activities in the primary screening agar diffusion assay, a modified microplate broth method according to Fyhrquist et al. (2014) and according to guidelines of the Clinical and Laboratory Standards Institute (2013) was used. *M. smegmatis* ATCC 14468 was grown on Löwenstein-Jensen agar slants for five days at +37°C. A colony from the agar slant was transferred into Dubos broth (Difco) and the turbidity of the sample was measured at 625 nm using a spectrophotometer (UV-Visible Spectrophotometer, Pharmacia LKB-Biochrom 4060). The absorbance at 625 nm was adjusted to 0.1 (approx. 1.0×10^8 CFU/ml) using Dubos broth (Difco).

This suspension was diluted further so that the final number of cells in the inoculum was approximately 5.0×10^5 CFU/ml. 100 µl of this inoculum was added to the wells of the microplate (Nunc, Nunclone, Denmark). In addition 100 µl of two-fold dilutions of the plant extracts (9.76-5000 µg/ml) and of rifampicin (0.98-1000 µg/ml) in Dubos broth or 100 µl broth (growth control) were added to the wells of the microplate, so that the final volume in each well was 200 µl. Thus, at the starting point of the experiment each well contained an inoculum of 2.5×10^5 CFU/ml. Plant extracts, fractions, pure compounds or rifampicin in broth without bacterial inoculum were used as sample controls. MeOH and hexane were used as solvent controls and did not affect the growth of *M. smegmatis* at 5 % (v/v) or less. The plates were incubated for four days at 37 °C, whereafter turbidity of the wells at 620 nm was measured using a Victor 1420 microplate reader (Wallac, Finland). As described in the below formula, the results were measured as mean percentage inhibition compared to the growth control of three replicate samples ± SEM. The minimum inhibitory concentration (MIC) was considered as the smallest concentration of the extracts, fractions, pure compounds and rifampicin inhibiting visible growth which was found to be equal to 90 % inhibition or more of the growth when measured spectrophotometrically at 620 nm.

% inhibition of growth (IC) = 100 (% growth of the growth control) – $((GT_{A620} - SC_{A620})/GC_{A620}) \times 100)$

where GT_{A620} is the turbidity of the test well at 620 nm (plant sample, pure compound or antibiotic), SC_{A620} stands for the test sample negative control and GC_{A620} is the turbidity of the growth control at 620 nm (containing only bacterial cells).

2.5. Statistical analysis

The data from diameters of inhibition and the percentage inhibition of growth were expressed as mean ± SEM, obtained from three independent experiments, each sample performed in duplicate, triplicate or quadruplicate.

3. Results and discussion

3.1. General observations

A total of seventy-seven extracts from the roots, stem bark, stem wood, fruits and leaves as well as three chromatographic fractions from the roots of *T. laxiflora* and *T. brownii* were tested for their growth inhibitory effects against *Mycobacterium smegmatis* ATCC 14468. The roots of both species of *Terminalia* gave the best growth inhibitory effects, although nearly all extracts

gave some activity, and growth inhibition was observed both for polar and non-polar extracts (Table 1 and 2). Our results on the good growth inhibitory effects of especially the root extracts of both species of *Terminalia* are in accordance with Mann et al. (2008) who reported that root extracts of *Terminalia avicennoides* give strong antimycobacterial effects.

3.2. Growth inhibition of medium-polar to polar extracts from various plant parts

Polar to medium polar extracts from the roots (methanol, acetone and ethyl acetate) of *T. laxiflora* gave lower MIC values (625-2500 µg/ml) than root extracts of similar polarities from *T. brownii* (MIC 2500-5000 µg/ml) (Table 2), although the zones of inhibition were larger for the root extracts of *T. brownii* (Table 1). Interestingly, cold water extracts (macerations) and decoctions of the roots of both species gave good antimycobacterial effects in terms of the sizes of their inhibition zones (Table 1). Notably, when using methanol Soxhlet extraction, the extraction yield for the root bark was especially beneficial for *T. brownii*, giving a 78.2% yield compared to 28.8% and 43.4% yields, respectively, for a wood and a stem bark extract (Fig. S8 a). For *T. laxiflora*, however, the roots gave smaller extraction yields (34%) than the stem bark (41.1%) when using Soxhlet methanol extraction (Fig. S8 a). For both species of *Terminalia*, the yields of decoctions and macerations of the roots were lower than the yields obtained with methanol Soxhlet extraction (Fig. S8 b) which means that the traditional procedure of preparing these plants for medicine gives a fairly low yield of antimycobacterially active compounds. Our results justify the traditional uses of the roots of *T. laxiflora* for treatment of tuberculosis and cough (Foyet and Nana, 2013), but we suggest that ethanol extracts could be used instead of macerations and decoctions. The roots of *T. brownii* are only reported to be used for allergic reactions (Mosango, 2013), and therefore our results now suggest that the roots of this species also could have uses for the treatment of TB.

When compared to the root extracts, the extracts of stem wood and bark of both species of *Terminalia* in general gave smaller zones of inhibition (Table 1). Interestingly, decoctions of the stem wood of *T. brownii* and *T. laxiflora* gave good growth inhibition whereas decoctions of the bark of both species showed only slight activity. Thus, our results justify the use of decoctions and infusions of the stem wood of *T. brownii* for treatment of fevers, colds and chest complaints, including tuberculosis (Burkil, 1994). Here must be noted that fairly low yields of 2.8 and 6 % resulted from hot water extraction of the stem wood of *T. brownii* and *T, laxiflora*, respectively (Fig. S8 b). Thus, again, ethanol might be a better solvent for the preparation of traditional medicine from the stem wood of these species to treat TB, since the yields resulting from methanol Soxhlet extraction were higher than for hot water extraction (Fig. S8 a).

In addition to the roots and stem also the leaves of *T. brownii* and *T. laxiflora* contain antimycobacterial compounds and we found that ethyl acetate extracts of the leaves of both species gave large zones of inhibition (Table 1). We found, however, that the yields resulting from ethyl acetate extraction, used as one of the solvents in our sequential extraction procedure, were rather low for both species of *Terminalia* (Fig. S9 a and S9 b)

We hypothesize that the variation in antimycobacterial potency between extracts of different plant parts of *T. laxiflora* and *T. brownii* could be due to the *ratios* of the various polyphenolic compounds, especially that of ellagitannins in these organs, so that the optimum ratio might be present in the roots which gave the most prospective growth inhibitory effects in our study. Roots in general have been found to contain higher levels of antimicrobial defense compounds when compared to other organs (Balmer and Mauch-Mani, 2013). It is possible that some of the compounds in the roots of *T. laxiflora* and *T. brownii* (ellagitannins and other phenolic compounds) act synergistically with each other to maximize the defense capacity of the roots against pathogenic soil bacteria and fungi (Balmer and Mauch-Mani, 2013).

Leaf, stem bark and root extracts of *T. laxiflora* and *T. brownii* have been reported to give growth inhibitory effects against some gram-negative and gram-positive bacteria (Abd alla et al., 2013; Machumi et al., 2013; Mbwambo et al., 2007; Fasola et al., 2013; Muddathir and Mitsunaga, 2013; Salih et al., 2017), but this is the first report on their good antimycobacterial effects.

3.2.1. Growth inhibition of an ellagitannin enriched RP-18 TLC fraction from an ethyl acetate extract of the roots of T. laxiflora

Based on good growth inhibitory results from the primary screening, an ethyl acetate extract of the roots of *T. laxiflora* was chosen for further fractionation using preparative reversed phase thin layer chromatography (RP-18 TLC) in an attempt to separate antimycobacterial fractions and compounds from this extract, with special emphasis on ellagitannins. We have previously reported that the crude ethyl acetate extract of the roots of *T. laxiflora* contains a high concentration and diversity of unknown ellagitannins, along with punicalagin, corilagin and its derivative (Salih et al., 2017; Table S3). Muddathir et al. (2013) reported on the presence of flavogallonic acid dilactone and terchebulin in a methanolic extract of the stem wood of *T. laxiflora*, but we found that ethyl acetate extracts of the stem wood and roots of *T. laxiflora* were devoid of these ET:s.

Our TLC fractionation of an ethyl acetate extract of the roots of *T. laxiflora* resulted in altogether seven fractions (Fig. S1). Of these fractions, only fractions 5 (R_5) and 6 (R_6) were

available in high enough concentrations to be tested for their antimycobacterial effects. These fractions 5 (R_5) and 6 (R_6), showed a significant enrichment of ellagitannins, and especially in punicalagin, compared to the ethyl acetate extract (Table S3). Since fractions 5 (R_5) and 6 (R_6) were very similar to each other, both containing punicalagin as the main compound, only the other one (fraction 5) was investigated for its antimycobacterial effects. The MIC of fraction 5 (R_5) was 500 µg/ml and thus 2.5 times lower than the MIC (1250 µg/ml) for the crude ethyl acetate extract (Table 2). The compounds in the thin layer chromatography fraction 5 (R_5) were tentatively identified using HPLC-DAD data, comparing retention times and UV_{λ} max data to the corresponding compounds in the crude ethyl acetate extract (Table S3). According to this data, fraction 5 (R_5) contained a much higher concentration of punicalagin **(5)**, exhibiting an HPLC-DAD peak area of 29.62 % compared to a peak area of 12.99 % for punicalagin in the crude ethyl acetate extract (Table S3). Therefore, most of the antimycobacterial potential of fraction 5 is suggested to be attributed to punicalagin. Punicalagin has been found to totally inhibit the growth of *M. tuberculosis* typus humanus at concentrations higher than 600 µg/ml as well as to inhibit the growth of a patient strain of *M. tuberculosis* at concentrations higher than 1200 µg/ml and was the first ellagitannin reported to possess antimycobacterial effects (Asres et al., 2001). Punicalagin contains two galloyl groups, the number and position of which are known to be related to the antibacterial potential of ellagitannins (Shimozu et al., 2017). Davidiin, a small ellagitannin from *Davidia involucrata*, containing three galloyl groups, was found to be highly antibacterial against MRSA and VRE (MIC 16-64 µg/ml), compared to ellagitannins containing only hexahydroxy- (HHDP) and dehydrohexahydroxydiphenoyl (DHHDP) groups but lacking galloyl groups (Shimozu et al., 2017). Moreover, ellagitannin enriched extracts have been found to disrupt the membrane function in bacteria, eventually resulting in cell lysis (Shimozu et al., 2017; Todorovic et al., 2017).

We found that in fraction 5 (R_5), corilagin **(4)** (R_f value 0.549; Fig.S3) occurred in relatively higher concentrations, giving a peak area of 7.31 % compared to 6.69 % in the crude ethyl acetate extract of *T. laxiflora* (Table S3). Therefore, we investigated if this ellagitannin gives growth inhibitory effects against *M. smegmatis*. We found, however, that corilagin gave moderate inhibitory effects against *M. smegmatis* with a rather high MIC value of 1000 µg/ml (Table 2). According to the discovery that the number and position of galloyl groups are important to the antibacterial activity of polyphenols (Taguri et al., 2004; Shimozu et al., 2017), already mentioned, corilagin should be more antibacterial than punicalagin since it contains one more galloyl group than punicalagin. However, this seems not to be true for mycobacterial strains, according to the MIC results for punicalagin (MIC >600 µg/ml) reported by Asres et al. (2001) and our result for

corilagin (MIC 1000 µg/ml). On the other hand Asres et al. (2001) used *M. tuberculosis* for their test which differs from *M. smegmatis* in its sensitivity. We have not found any results in the literature on the growth inhibitory effects of punicalagin against *M. smegmatis* and punicalagin was not available for testing against *M. smegmatis* in our laboratory. Corilagin has been reported to be moderately growth inhibitory against gram-positive bacteria, such as *Helicobacter pylori* and *S. aureus* with MIC values from 128-500 µg/ml (Shimamura et al., 2016; Funatogawa et al., 2004; Burapadaja and Bunchoo, 1995). Moreover, corilagin was found to interfere with the activity of penicillin binding protein 2a (PBP2a) in *S. aureus*, but the mechanism of action remains unclear (Stapleton and Taylor, 2002). In addition, corilagin was found to increase the effects of β-lactams against MRSA (Shimizu et al., 2001). It is therefore possible that corilagin also might act synergistically together with rifampicin against *mycobacteria* and this remains to be investigated.

It has been suggested that the antioxidative effects of phenolic compounds might be related to their antimicrobial effects so that phenols reduce the production of stress related defense compounds in microorganisms and thus their pathogenicity (de Freitas Araujo et al., 2017; Dambolena et al., 2011). Moreover, plant extracts enriched with antioxidative compounds may treat symptoms of inflammation in the host organism due to microbial infections (Courtney et al., 2015). Therefore, we investigated the reducing capacity of compounds (ET:s and other polyphenols) in the crude ethyl acetate extract of *T. laxiflora* using a TLC-DPPH qualitative method (Fig. S1 D). In addition we compared a decoction to the ethyl acetate extract, since this preparation is frequently used in traditional medicine (Fig. S2 D). Moreover, the R_f values and reducing capacity of the pure standard compounds of corilagin, gallic acid and ellagic acid were compared to the corresponding compounds in the crude extracts (Fig. S3). We observed that several compounds in the ethyl acetate extract and in the decoction gave strong antioxidative effects as demonstrated by a colour change of the DPPH reagent from purple to yellow for antioxidative compounds on the thin layer plates (Fig. S1, S2 and S3). Strong antioxidative effects were observed for corilagin, ellagic acid and gallic acid in the crude ethyl acetate extract and the decoction (Fig. S1, S2 and S3). Our results are in accordance with Courtney et al. (2015) who report on both good antimicrobial and antioxidative effects of ellagitannin enriched extracts of *Terminalia ferdinandiana*, although these two properties were not correlated to each other in their report. Especially corilagin has been reported to give good antioxidative effects with an IC_{50} value of 53 µg/ml (Anokwuru et al., 2015). Therefore, we suggest that the antimycobacterial properties of decoctions of *T. laxiflora* used in traditional medicine, could be due to ellagitannins and partly via their antioxidative effects.

In conclusion, our results justify the use of standardized water extracts and decoctions of *T. laxiflora*, enriched with ellagitannins, for the treatment of tuberculosis in African traditional medicine. In Africa all parts of *T. laxiflora* are used for treatment of TB and cough (Foyet and Nana, 2013), and our results demonstrate now that especially the roots should be used for preparation of traditional medicine for treatment of TB. Care has to be taken when using extracts enriched with punicalagin, however, since high concentrations of punicalagin have been found to cause liver necrosis in mice (Doig et al., 1990).

3.2.2. Growth inhibition of Sephadex LH-20 fractions of a root extract of T. brownii

Sephadex LH-20 fractionation of a methanolic root extract of *T. brownii* was used to study the effects of ellagitannin and ellagic acid derivative enrichment on the MIC against *M. smegmatis*. Two fractions resulting from this purification, an acetone and an ethanol fraction (Fig. 1), were tested for their growth inhibitory effects against *M. smegmatis*. Our results demonstrate that, in comparison to the crude methanol extract which gave a MIC of 5000 µg/ml, the acetone wash, enriched with ellagitannins, gave a MIC of 62.5 µg/ml (Table 2, Fig.1B). Sephadex LH-20 purification thus results in a significant increase in antimycobacterial activity of the acetone soluble fraction. Moreover, in comparison, the MIC for rifampicin using microplate dilution method was 3.9 µg/ml (Table 2). We used HPLC-DAD analysis for tentative identification of compounds in the Sephadex LH-20 fractions. Retention times and UVλ absorption maxima of the compounds in the fractions were compared to compounds in the crude methanolic Soxhlet extract. We found that the acetone wash was enriched with methyl (*S*)-flavogallonate (**9)** (peak area % 14.30, Rt 12.37 min) and its isomer **(5)** (peak area % 12.21, Rt 8.56 min) as well as an unknown ellagitannin **(10)** at Rt 15.08 min (peak area % 25.64) (Fig. 1B and Table S4). Therefore, the antimycobacterial activity of the acetone wash is suggested to be mostly attributed to these ellagitannins. This fraction also contained a high number of other, unknown polar ET:s (Rt 8.04-13.94 min). These ellagitannins might act synergistically with each other to improve the antimycobacterial effects of this fraction.

Methyl *(S)*-flavogallonate has been reported to occur in various parts of other *Terminalia* species, such as in the leaves of *T. myriocarpa* (Marzouk et al., 2002) as well as in the fruits of *T. chebula*, *T. bellerica* and *T. horrida* (Pfundstein et al., 2010) and in the galls of *T. chebula* (Manosroi et al., 2013). Methyl *(S)*-flavogallonate contains gallic acid moieties esterified to a flavogallonoyl unit (Marzouk et al., 2002). Also castalagin, which is structurally related to methyl (*S*)-flavogallonate, contains a flavogallonoyl unit participating in the C-glycosidic linkage (Yoshida et al., 1992). Castalagin has been found to be strongly antibacterial against *S. aureus*, *Salmonella*

and *E. coli* (Taguri et al., 2004). There are no SAR studies, however, on the relationship between the occurrence of flavogallonoyl units in ellagitannins and their antimicrobial potential. Taguri et al. (2006) studied the relationship between structure and antimicrobial activity of polyphenols and they found that polyphenols containing pyrogallol (3,4,5-trihydroxyphenyl) groups are especially active. This would apply to methyl (*S*)-flavogallonate which contains four pyrogallol units. To the best of our knowledge the antimycobacterial effects of methyl (*S*)-flavogallonate has not been evaluated. Our results warrant further studies on the growth inhibitory effects of purified methyl (*S*)-flavogallonate, its isomer as well as of the unknown ellagitannin at Rt 15.08 min (Table S4 and Fig. 1; A, B and C).

We found that also the ethanol fraction (Fig. 1C) was more growth inhibitory than the crude methanol extract of the roots of *T. brownii* and gave a MIC value of 125µg/ml compared to 5000 µg/ml for the crude methanol extract (Table 2). We found that this fraction was enriched especially in ellagic acid **(14)** (Table S4**)**. Moreover, the isomer of methyl *(S)*-flavogallonate **(5)** was also found to be present in higher concentrations than in the acetone wash (Table S4, Fig. 1C). In addition the ethanol wash contained an acetylated ellagic acid derivative **(18)** and methyl ellagic acid xyloside **(13)**, which were absent in the acetone wash (Table S4 and Fig. 1C). Some ellagitannins with long retention times of 28.3 **(16)**, 28.9 **(17)** and 35.7 min **(19)** (two ET:s in one peak) were found in the ethanol fraction but were absent from the acetone fraction (Fig. 1, Table S4). On the other hand the unknown ellagitannin **(10)** at retention time 15.6 min was present in significantly smaller concentration (8.09% peak area) in the ethanol wash compared to the acetone wash (25.64% peak area) (Table S4, Fig. 1; B and C). It seems from our result that the acetone wash contains the optimal ratio of this ellagitannin **(10)** together with methyl *(S)*-flavogallonate **(9)** and its isomer **(5),** which are suggested to be important for strong antimycobacterial activity. The good growth inhibitory effects of the ethanol wash is instead suggested to be due to a combination of ellagitannins and ellagic acid derivatives.

Ellagic acid derivatives have been found to give good growth inhibitory effects against *M. tuberculosis* and *M. smegmatis*: Kuete et al. (2010) found that 3,4′-di-*O*-methylellagic acid 3′-*O*-β-D-xylopyranoside from the stem bark of *T. superba* inhibited the growth of *M. smegmatis* and a panel of *M. tuberculosis* strains with MIC values ranging from 4.88-39 µg/ml, and the MIC values were lower than those of isoniazid against some clinical strains of *M. tuberculosis*. In contrast to this result we found that ellagic acid gave a MIC of 500 µg/ml against *M. smegmatis* (Table 2). Therefore, it seems that ellagic acid should be combined with a sugar molecule and/or possess methyl groups to become more active. We suggest that the good growth inhibitory effects of the

ethanol fraction of the roots of *T. brownii* might be due to ellagic acid xyloside and methyl ellagic acid xyloside. Ellagic acid derivatives and ellagic acid itself have been found to bind to proteins and in this way inactivate microbial adhesions, membrane transport proteins and enzymes (Haslam, 1996). Ellagic acid derivatives such as 3,3′-di-*O*-methylellagic acid is also known to interfere with the biosynthesis of mycolic acid, the main constituent of the mycobacterial cell wall (Kondo et al., 1979). Ellagic acid derivatives are considered to be important models for the development of new future anti-TB drugs since molecular docking studies on the effects of ellagic acid derivatives on enzymes important to the mycobacterial cell wall biogenesis have revealed ellagic acid based molecules, such as pteleoellagic acid, with promising activities (Shilpi et al., 2015).

Our results show that *Terminalia brownii* could be a good source of new ellagic acid derivatives and ellagitannins with antimycobacterial potential and more studies are warranted on the antimycobacterial activity of isolated ellagic acid derivatives and ellagitannins from this species.

3.3. Growth inhibition of lipophilic extracts

As shown in Table 1, the hexane extracts of the stem bark and stem wood and the dichloromethane extracts of the root of *T. laxiflora* and *T. brownii* demonstrate good growth inhibition against *M. smegmatis*, showing inhibition zones of 19.5-21.3 mm. This result supports the traditional uses of hot water decoctions of the stem bark and wood of *T. brownii* and *T. laxiflora* against cough (El Ghazali et al., 2003 and Mohieldin et al 2017), since decoctions also contain non-polar compounds such as triterpenes and free fatty acids which we have found to be present in the hexane extracts of these species (Table 5, Fig. 2, 3 and S7). Here must be noted briefly, that the yields of extraction of hexane extracts of the stem bark and wood of both *T. brownii* and *T. laxiflora* is typically very low, compared to yields given by more polar solvents such as methanol (Fig. S8 a). Thus, it might be difficult to isolate antimycobacterial compounds from these extracts. In contrast to the hexane extracts of the stem bark and wood of *T. laxiflora* and *T. brownii*, we observed no growth inhibitory activity for the hexane extracts of the leaves of these species (Table 1).

Our GC/MS analysis of the antimycobacterially active hexane extracts of the stem bark of *T. laxiflora* and *T. brownii*, resulted in the identification of thirty compounds, among which five triterpenes, three steroids, four long-chain fatty acids and three fatty alcohols were characterized (Table 5, Fig. 2 and 3). The similar qualitative composition might explain the similarities we have observed in antimycobacterial activities for the two hexane extracts of the stem bark of *T. laxiflora* and *T. brownii*, both giving a MIC value of 2500 µg/ml (Table 2, Fig. 2).

The following long-chain fatty acids were characterized for the first time in *T. laxiflora* and *T. brownii* and have to the best of our knowledge not previously been detected in the genus *Terminalia* (Table 5 and Fig. 2, 3 and S7); **(1)** 1,18-octadec-9-ene-dioate (C18:1), **(2)** tetracosanoic acid (syn. lignoceric acid) (C24:0), **(3)** hexacosanoic acid (syn. cerotic acid) (C26:0) and **(5)** octacosanoic acid (C28:0). Unsaturated fatty acids are known to possess antibacterial (Zheng et al., 2005) and antimycobacterial effects, such as linoleic acid, which gave a MIC of 2 µg/ml against *Mycobacterium aurum* (Seidel and Taylor, 2004). The growth inhibitory activities of fatty acids have been found to vary with chain length and degree of unsaturation, so that chain lengths of 14 C-atoms seem to be optimal and a high degree of unsaturation renders fatty acids more active (Kondo and Kanai, 1977; Luo et al., 2011; Seidel and Taylor, 2004). The unsaturated fatty acid (**1**), containing one double bond, was present in high concentrations especially in *T. brownii*, but also in *T. laxiflora* (Table 5 and Fig. 2, 3 and S7) and is therefore suggested to account for some of the antimycobacterial effects we have seen in hexane extracts of these species. The other saturated fatty acids (**2**), (**3**) and (**5**) present in *T. brownii* and *T. laxiflora* are suggested to be less active accordingly. We tested the growth inhibitory activity of some saturated fatty acids, such as stearic (C18:0) and behenic acid (C22:0), which we have found to occur in chloroform and dichloromethane extracts of the stem bark of both *Terminalia* species and which we assume to be present in minute quantities also in the hexane extracts, and found that they did not give any growth inhibition at concentrations of 1000 µg/ml (Table 2). These fatty acids are also known to be present in other species of the genus *Terminalia* (de Gouveia Baratelli et al., 2012; Kim et al., 2006; Onial et al., 2014). Our results on behenic and stearic acid antimycobacterial activity supports earlier findings that saturated fatty acids with chain lengths shorter or longer than C14:0 are not antimycobacterial (Kondo and Kani, 1977; Seidel and Taylor, 2004).

To the best of our knowledge research on fatty acids from native African *Terminalia* species is scanty, but there is a rich body of literature concerning fatty acid composition in *Terminalia chebula* seed oil (Janporn et al., 2015; Onial et al., 2014; Hosamami, 1994). No literature on the antimycobacterial effects of these fatty acids does exist, however. The amphiphilic architecture of fatty acids, consisting of a polar head group and flexible hydrophobic tail, make them an interesting group of antimycobacterial molecules, killing mycobacteria by inhibiting the biosynthesis of mycolic acid, an important constituent of the mycobacterial cell wall (Rugutt and Rugutt, 2012; Zheng et al., 2005). Therefore, further research on the antimycobacterial effects of fatty acids isolated from *T. brownii* and *T. laxiflora* are warranted. Owing to the almost certain presence of fatty acids in hot water decoctions of these species, the frequently used traditional

preparation of these plants, it would be important to test individual fatty acids in these decoctions for antibacterial and antimycobacterial potency.

The fatty alcohols octacosanol **(4)**, triacontanol **(6)** and dotriacontanol **(10)** were reported for the first time in the stem bark of *T. laxiflora* and *T. brownii* (Table 5 and Fig. 2, 3 and S7). Octacosanol (**4**) and triacontanol (**6**) occurred in higher concentrations in *T. laxiflora* than in *T. brownii* (Table 5 and Fig. 2). We investigated the growth inhibitory effects of pure triacontanol which gave a MIC value of 250 µg/ml against *M. smegmatis* (Table 2). Although the stem bark of *T. laxiflora* contained a higher concentration of triacontanol than the stem bark of *T. brownii*, the n-hexane extracts of both species gave the same MIC value of 2500 µg/ml against *M. smegmatis*. This means that the precise concentration of triacontanol might not be critical for the antimycobacterial activity of these extracts, but maybe merely the presence of this compound, in order to contribute to the overall activity of the extracts. Also, it must be emphasized here, that two different methods, the microplate dilution and the agar diffusion were used for the pure compounds and extract, respectively, thus explaining the high relative differences in the MIC values. Some plant derived fatty alcohols are known to give potent antimycobacterial effects, such as phytol, which gave a MIC value of 2 µg/ml against *M. tuberculosis* H37Rv (Rugutt and Rugutt, 2012).

We characterized β-sitosterol **(7)**, stigmast-4-en-3-one (sitostenone) (**9**) and 5α-stigmastan-3,6-dione (**13**) from the hexane extracts of the stem bark of *T. laxiflora* and *T. brownii*, by comparison of their mass spectra with Wiley Natural Library and standard compounds (Table 5 and Fig. 3; f, h and i). To the best of our knowledge 5α-stigmastan-3,6-dione **(13)** has not been previously found in the genus *Terminalia* and moreover, antimycobacterial potency of this compound has not been elucidated. β-sitosterol was present in significantly higher concentrations in *T. brownii* when compared to *T. laxiflora* (Table 5 and Fig. 2). We found that β-sitosterol as well as the chemically closely related stigmasterol, the later of which we have found in the leaves of *T. brownii* and *T. laxiflora*, both gave a MIC of 500 µg/ml against *M. smegmatis* (Table 2). β-sitosterol from hexane extracts of *Morinda citrifolia* showed anti-tubercular activity with a MIC of 128 µg/mL against *M. tuberculosis* H37Rv (Saludes et al., 2002). These differences in MIC might be explained by the uses of different mycobacterial strains, *M. tuberculosis* H37Rv being more sensitive than *M. smegmatis* against some antimycobacterial agents (Zhang et al., 2003). A recent study by Edilu et al. (2015), demonstrated large inhibition zones of β-sitosterol isolated from *Caylusea absyssinica* (Resedaceae) against a wide range of bacteria, among them *Salmonella typhimurium* and *Pseudomonas aeruginosa*.

Earlier, β-sitosterol has been found in the root bark of *T. laxiflora* (Ekong and Idemudia, 1967) and stem bark of *T. brownii* (Machumi et al., 2013; Opiyo et al., 2011), and in the fruit of *Terminalia glaucescens*, *T. ivorensis*, *T. chebula* and *T. phanerophlebia* (Bulama et al., 2015; Bag et al., 2013; Nair et al., 2012; King et al., 1955). Recently, stigmasterol has been discovered in *Terminalia mantaly* (Tchuenmogne et al., 2017). Moreover, β-sitosterol and sitostenone have been described in the stem bark of *Terminalia sericea* (Nkobole et al., 2011). Sitostenone from *Rhizoctonia solani* gave antibacterial activity with half maximal inhibitory concentration (IC_{50}) of 109.9 µg/mL against *Pseudomonas lachrymas* (Liang et al., 2015).

According to the mass fragmentation pattern and comparing to the Wiley Natural Library, standard compounds and to Goad and Akihisa (1997) and Assimopoulou and Papageorgiou (2005), a total of five triterpenes were characterized from the hexane extracts of the stem bark of *T. brownii* and *T. laxiflora* (Table 5 and Fig. 2, 3g and S7); β-amyrine (**8**), friedelin (syn; friedelin-3-one, friedelanone) **(11),** betulinic acid (**12**) and two oleanane-type triterpenoids **(14)** and (**15**). Friedelin was quantitatively the main compound in hexane extracts of both *Terminalia* species (Table 5 and Fig.2, 3g and S7). There are no previous reports on the occurrence of friedelin in *T. brownii* and *T. laxiflora*. Friedelin has been found in various parts of other species of *Terminalia*, such as the stem bark of *Terminalia mollis* (Liu et al., 2009) and the trunk bark of *T. glabrescens* (Garcez et al., 2003). Friedelin, isolated from the root bark of *Terminalia avicennioides*, gave a MIC of 4.9 µg/ml against the *Bacillus Calmette Guerin* tuberculosis vaccine, consisting of weakened *Mycobacterium bovis* (Mann et al., 2008; Mann et al., 2011). In another investigation friedelin gave a MIC of 128 µg/ml against *M. tuberculosis* (Higuchi et al., 2008b). In our screening, however, we found that friedelin only moderately inhibited the growth of *M. smegmatis* with a MIC of 250 µg/ml (Table 2) and this rather high MIC might again be due to *M. smegmatis* being relatively resistant in comparison to *M. tuberculosis* and *M. bovis*. This would, in general, apply to that a low MIC against *M. smegmatis* would result in even a lower MIC against *M. tuberculosis*. Besides, friedelin has been found to give broad spectrum antibacterial effects against both gram-positive and gram-negative bacteria with MIC values ranging from 2.44 -78.12 µg/ml (Kuete et al., 2007; Wang et al., 2014). We suggest that friedelin might contribute significantly to the antimycobacterial effects of the hexane extracts of *T. brownii* and *T. laxiflora*.

β-amyrine (**8**) has been identified in other *Terminalia* species, such as *T. ivorensis* (King et al., 1955) and *T. glaucescens* (Atta-ur-Rahman et al., 2002). β-amyrine, isolated from a chloroform fraction of the leaves of *Byrsonima fagifolia* gave potent antitubercular activity with a MIC of 31.5 µg/mL against *M. tuberculosis* H37Rv (Higuchi et al., 2008a). Therefore, even though

β-amyrine is present only in small quantities in the hexane extracts of the stem bark of *T. brownii* and *T. laxiflora* (Fig. 2, S7), this triterpene could still contribute to the good overall antimycobacterial effect of these extracts.

We found that betulinic acid (**12**) was present in minute quantities in *T. laxiflora* and *T. brownii* (Fig. 2). Betulinic acid has previously been described in the stem bark of *T. brownii* and was found to inhibit the growth of *Streptomyces ipomaea* (Opiyo et al., 2011). To the best of our knowledge, this is the first report on the occurrence of betulinic acid in *T. laxiflora*. Betulinic acid is also known from the barks of *Terminalia catappa* (Pertuit et al., 2015) and was found to give some growth inhibition against *M. tuberculosis* with a MIC of 400 µg/ml and an IC50 of 84µg/ml (Li et al., 2015). Therefore, betulinic acid might contribute to the antimycobacterial activity of the hexane stem bark extracts of *T. laxiflora* and *T. brownii.*

Lastly, we found two oleanane-type triterpenes (**14** and **15**) in the hexane extracts of the stem bark of *T. brownii* and *T. laxiflora* (Table 5 and Fig. 2). To the best of our knowledge oleanane-type triterpenoids have not been reported before in *T. laxiflora*. However, a recent study by Machumi et al. (2013), has reported on the occurrence of an oleanane-type triterpenoid in the stem bark of *T. brownii*. In addition, several authors have reported on oleanane-type triterpenoids in other *Terminalia* species (Elsayed et al., 2015; Zhang et al., 2015; Aiyelaagbe et al., 2014; Kakoli et al., 2005; Garcez et al., 2003; Anjaneyulu et al., 1986). Oleanolic acid from *Lantana hispida* inhibited the growth of *M. tuberculosis* with a MIC of 25 µg/ml (Jesus et al., 2015). Oleanane-type triterpenoids were found to affect peptidoglycan metabolism in *Listeria monocytogenes* (Kurek et al., (2010). Morover, oleanane-type triterpenes have been found to suppress enzymatic function in the mycobacterial cell and to increase free radical molecules (Podder et al., 2015 and López-García et al., 2015). Generally, lipophilic terpenoids affect the membrane function (Aleksic and Knezevic, 2014; Mochizuki and Hasegawa, 2006). Therefore, the two oleanane-type triterpenes (**14** and **15**) we have found in *T. brownii* and *T. laxiflora* should be investigated for their cell wall synthesis inhibiting and membrane affecting properties in mycobacterial species. Peptidoglycan is a highly interesting target for new antimicrobials.

4. Conclusions

We hereby report on the chemical structures of forty-five compounds from the stem bark, stem wood and the roots of *T. laxiflora* and *T. brownii*. Ellagitannins, ellagic acid and its derivatives as well as fatty acids, fatty alcohols, steroids and triterpenes present in extracts of these species could have potential as antimycobacterial leads and TB drug adjuvants. Our results support the traditional uses of *T. brownii* and *T. laxiflora* as decoctions for treatment of tuberculosis and

cough. Therefore, further research is needed on optimization and standardization of the phytochemical composition of extracts of the studied species for more effective and safer use in traditional medicine.

Conflict of interest

The authors have no conflict of interest to proclaim.

Appendix A. Supplementary materials

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Fig. 1. Crude MeOH Soxhlet extract of the root of *Terminalia brownii* (A) and its Sephadex-LH 20 fractions (B and C). Gallic acid **(1)**; unknown ellagitannin $[M-H]^-$ 456.9961 **(2)**; unknown ellagitannin **(3)**; unknown ellagitannin **(4)** and isomer of methyl-S-flavogallonate **(5)**; gallotannin **(6)**; ellagitannin **(7)**; ellagitannin **(8)**; methyl-(*S*)-flavogallonate **(9)**; ellagitannin $[M-H]^-$ 609.1088 **(10)**; ellagic acid glucuronide **(11)**; ellagic acid xyloside **(12)**; methyl ellagic acid xyloside **(13)**; ellagic acid **(14)**; trimethyl ellagic acid **(15)**; ellagitannin $[M-H]^-$ 725.4141 **(16)**; ellagitannin $[M-H]^-$ 817.4003 **(17)**; acetylated ellagic acid derivative **(18)**; two ellagitannins at $[M-H]^-$ 577.1369 and 577.1392 **(19)**; ellagitannin $[M-H]^-$ 817.3999 **(20)**.

Fig. 2. GC/MS chromatogram of fatty acids and triterpenes in a hexane extract of the stem bark of (A) *T. laxiflora* and (B) *T. brownii.* TMS-1,18-octadec-9-ene dioate **(1)**;TMS-tetracosanoic acid **(2)**; TMS-hexacosanoic acid **(3)**; TMS-octacosanol **(4)**; TMS-octacosanoic acid **(5)**; TMS-triacontanol **(6)**; TMS- β-sitosterol **(7)**; TMS-β-amyrine **(8)**; stigmast-4-en-3-one **(9)**; TMS-dotriacontanol **(10)**; TMS-friedelin **(11)**; TMS-betulinic acid **(12)**; 5α-stigmastan-3,6-dione **(13)**; TMS-oleanane-type triterpenoids **(14)** and **(15).**

Fig. 3 a-f. TMS mass spectra of compounds (**1), (2), (3), (4), (6) and (7)** in *T. brownii* and *T. laxiflora* (numbering according to Fig. 3 and Table 5). **Fig. 3 g-j.** TMS mass spectra for compounds (**9), (10),** (**11)** and (**13).**

Table 1 Growth inhibitory activity of extracts and fractions of various organs of *T. brownii* and *T. laxiflora* against *Mycobacterium smegmatis* ATCC 14468. Results obtained with an agar diffusion method.

Extracts, fractions and antibiotics	***T. laxiflora***		***T. brownii***	
	IZ	**AI**	**IZ**	**AI**
Root extracts				

R. EtOAc	18.5 ± 0.4	0.49	**20.3 ± 0.0**	**0.54**
R. Me*	18.7 ± 0.3	0.5	**22.0 ± 0.0**	**0.58**
R. acet	**20.7 ± 0.9**	**0.52**	**19.5 ± 0.5**	**0.52**
R. Dic	**21.0 ± 0.6**	**0.56**	**20.3 ± 0.3**	**0.54**
R. hex	17.0 ± 0.6	0.45	14.0 ± 0.0	0.37
R. aqu	19.3 ± 0.7	0.51	**23.3 ± 0.7**	**0.62**
R. H_2O*	**21.3 ± 0.9**	**0.57**	**26.7 ± 0.7**	**0.71**
R. HH_2O	**22.0 ± 0.6**	**0.58**	**21.7 ± 0.2**	**0.58**
R. MeSox	**22.3 ± 0.3**	**0.59**	**21.0 ± 0.6**	**0.56**
Rb. Dic	NT	NT	18.2 ± 0.4	0.48
Rb. EtOAc	NT	NT	**21.3 ± 0.9**	**0.57**
Rb. acet	NT	NT	**23.3 ± 0.9**	**0.62**
Rb. Me*	NT	NT	**20.0 ± 0.6**	**0.53**
Rb. MeSox	NT	NT	**17.0 ± 0.6**	**0.45**
Rb. HH_2O	NT	NT	19.3 ± 0.3	0.51
Rb. aqu	NT	NT	15.7 ± 0.7	0.42
Rb. H_2O*	NT	NT	**20.0 ± 0.6**	**0.53**
Rb. hex	NT	NT	13.7 ± 0.2	0.36
Sephadex LH-20 fractions from *T. brownii* roots:				
AW	NT	NT	**28.5 ± 0.1**	**0.76**
ET	NT	NT	**25 ± 0.0**	**0.66**
Stem wood extracts				
W. H_2O*	18.0 ± 0.0	0.48	15.7 ± 0.3	0.42
W. MeSox	16.7 ± 0.3	0.44	16.5 ± 0.3	0.44
W. EtOAc	16.3 ± 0.3	0.43	17.3 ± 0.3	0.46
W. HH_2O	18.0 ± 0.6	0.48	**21.3 ± 0.3**	**0.57**
W. hex	**20.3 ± 0.0**	**0.54**	**19.5 ± 0.5**	**0.52**
W. Ch	NA	NA	15.0 ± 0.0	0.40
W. Me*	17.0 ± 0.0	0.45	16.7 ± 0.7	0.44
W. aqu	14.0 ± 0.0	0.37	NA	NA
Stem bark extracts				
B. Me*	14.0 ± 0.0	0.37	16.0 ± 0.6	0.42
B. hex	**21.0 ± 0.6**	**0.56**	**21.3 ± 0.9**	**0.57**
B. aqu	13.5 ± 0.0	0.36	NA	NA
B. Ch	19.5 ± 0.5	0.52	**20.3 ± 0.3**	**0.54**
B. EtOAc	17.7 ± 0.7	0.47	16.3 ± 0.7	0.43
B. HH_2O	17.7 ± 0.3	0.47	13.8 ± 0.2	0.37
B. MeSox	13.8 ± 0.2	0.37	14.7 ± 0.7	0.39
B. H_2O*	14.0 ± 0.0	0,37	14.5 ± 0.0	0,38
Leaf extracts				
L. acet	**20.0 ± 0.6**	**0.53**	**27.0 ± 0.6**	**0.72**
L. Dic	NA	NA	14.8 ± 0.7	0.39
L. Me*	18.0 ± 0.0	0.48	**22.0 ± 0.3**	**0.58**
L. EtOAc	**23.0 ± 0.6**	**0.61**	**22.7 ± 0.7**	**0.60**

L. MeSox	18.0 ± 0.6	0.48	18.0 ± 0.6	0.48
L. hex	NA	NA	NA	NA
L. HH_2O	NA	NA	16.8 ± 0.4	0.45
L. aqu	16.0 ± 0.6	0.42	16.0 ± 0.6	0.42
Fruit extracts				
F. Me*	**20.0 ± 0.0**	**0,53**	**NT**	**NT**
F. HH_2O	NA	NA	**NT**	**NT**
Rifampicin	**37.7 ± 0.3**	**100**	**37.7 ± 0.3**	**100**

W, Stem wood; b, stem bark; R, roots; Rb, root bark; F, fruits; L, leaves; H_2O^*, cold water extracts (macerations); HH_2O, decoctions; MeSox, methanolic Soxhlet extract; Me*, cold methanol extracts; aqu, aqueous fraction; EtOAc, ethyl acetate fraction; acet, acetone fraction, Dic, dichloromethane fraction; hex, hexane fraction; Ch, chloroform fraction; Sephadex LH-20 fractions of a methanol extract of the roots of *T. brownii*: AW, acetone wash; ET, ethanol wash; AI, Activity index relative to rifampicin. Two hundred µl of extracts/fractions (50 mg/ml) and rifampicin (10 mg/ml) were applied on filter paper disks; IZ, Diameter of inhibition zones in mm as mean of triplicates (n = 3) ± SEM of three experiments. Most promising results (IZ ≥ 20mm) is indicated by bold text.

Table 2 Minimum inhibitory concentration (MIC) of extracts and of fractions obtained with Sephadex LH-20 and RP-18 TLC as well as pure compounds found in *T. laxiflora* and *T. brownii* against *Mycobacterium smegmatis* ATCC 14468. Results in µg/ml.

Extracts, fractions and antibiotic	***T. brownii***	***T. laxiflora***
Roots:		
Methanolic Soxhlet extract	5000	1250
Ethyl acetate extract	**2500**	**1250**
Cold water extract (Maceration)	5000	2500
Acetone extract	5000	**625**
Leaves:		
Acetone extract	2500	5000
Ethyl acetate extract	5000	2500
Bark:		
Hexane extract	2500	2500
Sephadex LH-20 fractions of *Terminalia brownii* root:		
Acetone wash	**62.5** (IC94)	NT

Ethanol wash	**125** (IC96)	NT
RP18-TLC fractions of *Terminalia laxiflora* root:		
1 R_5 EtOAc (Fraction 5)	NT	**500** (IC98)
Pure compounds found in the studied plants:		
Ellagic acid[R]	500 (IC_{98})	
Corilagin[R]	1000 (IC_{94})	
Friedelin[S]	250 (IC_{91})	
Triacontanol[S]	250 (IC_{89})	
Sitostenone[S]	250 (IC_{96})	
Stigmasterol[LS]	500 (IC_{98})	
β-Sitosterol [S]	500 (IC_{99})	
Stearic acid [S]	NA	
Behenic acid [S]	NA	
Rifampicin	39,06 (**3,90** IC_{98})	

NT, Not tested; MIC, in bold text for results obtained using microplate method; NA, not active at concentrations < 1000 µg/ml; (IC), the percentage growth inhibition resulting from MIC; [R] detected in roots of *T. laxiflora* and *T. brownii*; [S] detected in stem bark and wood of *T. brownii* and *T. laxiflora*; [L] present in leaf extracts; Stearic acid and behenic acid were present in dichloromethane and chloroform extracts of the stem bark and wood.

Table 5. GC/MS data of fatty acids, fatty alcohols, sterols and triterpenoids in hexane extracts of the stem bark of *T. laxiflora* and *T. brownii*.

Compound number	Compound name or Compound class	Molecular formula	Retention time	Molecular ion (M^+) and prominent fragment ions (m/z value) (relative intensity percentage)	Peak area % in *T. laxiflora*	Peak area % in *T. brownii*
(1)	1,18-octadec-9-ene-dioate-TMS	$C_{18}H_{32}O_4$-TMS	8.30	**456[M^+](2)** 441(17) 397(2) 366(5) 333(1) 276(12) 248(3) 217(12) 204(8) 147(25) 135(13) 133(19) 131(17) 129(100) 117(49) 95(34) 81(51)	**11.39**	**15.35**
(2)	Tertracosanoic acid 24:0-TMS	$C_{24}H_{48}O_2$-TMS	9.23	**440[M^+](9)** 425(26) 397(1) 381(1) 227(2) 201(6) 185(5) 170(3) 159(3) 145(38) 132(45) 129(59) 117(100) 97(13) 95(9) 85(13) 83(20)	1.22	1.15
(3)	Hexacosanoic acid 26:0-TMS	$C_{26}H_{52}O_2$-TMS	10.45	**468[M^+](9)** 453(19) 425(1) 409(1) 369(0,5) 297(0,8) 201(4) 185(4) 145(41) 132(51) 117(100) 97(16) 85(14) 84(6)83(23) 80(11)	1.14	0.62
(4)	Octacosanol (IOH-28:0), 28:0-TMS	$C_{28}H_{58}O$-TMS	11.18	**467[M^+](86)** 451(2) 153(1) 143(4) 139(2) 129(15) 115(15) 111(18) 103(100) 97(48) 90(41) 89(37) 85(52) 83(64) 81(23)	**3.72**	0.93
(5)	Octacosanoic acid 28:0-TMS	$C_{28}H_{56}O_2$-TMS	11.94	**496[M+](9)** 481(17) 453(1) 437(1) 283(1) 269(1) 255(1) 241(2) 201(5) 185(5) 159(2) 145(43) 133(17) 132(47) 129(56) 117(100) 83(25)	**2.34**	1.70
(6)	Triacontanol (IOH-30:0), 30:0-TMS	$C_{30}H_{62}O$-TMS	12.87	**495[M^+](100)** 479(2) 395(2) 381(0,6) 367(1) 353(1) 339(2) 325(0,7) 255(10) 185(4) 145(15) 133(16)) 129(50) 103(69) 91(47) 83(91)	**18.58**	**4.66**
(7)	β-Sitosterol-TMS	$C_{29}H_{50}O$-TMS	13.50	**486[M+](6)** 471(2) 396(12) 381(6) 357(15) 329(1) 255(6) 229(1) 213(5) 203(3) 201(3) 189(4) 161(11) 145(22) 131(20) 129(100) 105(35)	**2.93**	**6.51**
(8)	β-amyrine-TMS	$C_{30}H_{50}O$-TMS	14.14	**498[M+](6)** 484(4) 483(5) 410(2) 393(18) 279(2) 253(6) 241(6) 218(47) 203(16) 189(22) 161(17) 147(33) 133(38) 129(67) 95(86) 81(100)	**2.25**	**3.87**
(9)	Stigmast-4-en-3-one (Sitostenone)	$C_{29}H_{48}O$	14.84	**412[M^+](13)** 397(3) 355(3) 289(10) 271(2) 245(4) 229(26) 215(4) 201(5) 187(10) 161(10) 147(31) 133(24) 129(12) 124(100) 95(57) 81(55)	1.40	**2.89**
(10)	dotriacontanol (IOH-32:0), 32:0-TMS	$C_{32}H_{66}O$-TMS	14.97	**523[M^+](71)** 507(2) 485(2) 444(2)412(2) 381(1) 367(1) 355(1)341(2)325(2) 297(2) 281(5) 269(2) 253(3)129(20) 103(100) 83(72)	1.08	1.07
(11)	Friedelin (Friedelanone)-TMS	$C_{30}H_{50}O$-TMS	16.20	**426[M^+](4)** 411(2) 341(1) 302(4) 287(2) 273(9) 259(2) 257(2) 231(8) 218(9) 205(13) 163(18) 137(20) 123(48) 109(66) 95(99) 81(100)	**37.48**	**37.74**
(12)	Betulinic acid-TMS	$C_{30}H_{48}O_3$-TMS	16.32	**600[M^+](1)** 585(2) 510(1) 495(0,6) 482(2) 393(1) 385(2) 320(4) 281(4) 253(3) 215(6) 203(19) 189(47)175(19) 147(22) 129(100) 95(59)	**2.03**	1.27
(13)	5α-stigmastan-3,6-dione	$C_{29}H_{48}O_2$	16.96	**428[M^+](21)** 413(3) 399(4) 342(1) 330(2) 315(2) 299(1) 287(9) 273(3) 245(27) 191(11) 161(10) 149(17) 137(23) 131(100) 81(85)	0.79	1.53
(14)	TMS-Oleanane-type triterpenoid molecule		17.56	**570[M^+](2)** 555(4) 510(2) 495(0,8) 452(13) 393(2) 353(1) 320(13) 281(4) 255(3) 215(5) 203(100) 189(47) 161(8) 147(15) 133(30) 105(37)	**2.36**	1.53
(15)	TMS-Oleanane-type triterpenoid molecule		18.42	**570[M^+](1)** 555(3) 452(8) 393(1) 320(43) 307(10) 295(1) 281(3) 249(3) 203(100) 189(39) 173(18) 159(13) 145(20) 133(86) 105(41) 95(42)	**5.99**	**3.68**

Fig. S1. Thin layer chromatography finger print of ethyl acetate extract of the root of *Terminalia laxiflora*. (A) at visible light; (B) at 254 nm; (C) at 366 nm; (D) sprayed with DPPH reagent.

Fig. S2. Thin layer chromatography finger print of a decoction of the root of *Terminalia laxiflora*; (A) at visible light; (B) at 254 nm; (c) at 366 nm, (D) sprayed with DPPH reagent.

Fig. S3. Thin layer chromatography finger print of corilagin (R_f 0.549), ellagic acid (R_f 0.173) and gallic acid (R_f 0.721); (A) at 254 nm; (B) at 366 nm, (C) sprayed with DPPH reagent.

Fig. S7. Triterpenoid (11), sterol (8) and fatty acids (1 and 6) in hexane extracts of the stem bark of *T. laxiflora* and *T. brownii*.

Fig. S8. Percentage yield (% w/w) resulting from single solvent extractions with a polar and non-polar solvents. a) Methanolic Soxhlet and hexane extracts of *T. brownii* and *T. laxiflora*, b) Macerations and decoctions of *T. brownii* and *T. laxiflora*. TB, *Terminalia brownii;* TL, *Terminalia laxiflora;* R, roots; Rb, root bark; W, stem wood; B, stem bark; L, leaves; F, fruits; MeSox, methanolic Soxhlet extract; hex, hexane extract; HH2O, decoctions; H2O*, cold water extracts (macerations).

Fig. S9. Percentage yield (% w/w) resulting from sequential extraction of a) *T. brownii* and b) *T. laxiflora.* R, whole root; Rb, root bark; L, leaves; hex, hexane extract; Dic, dichloromethane extract; acet, acetone extract; EtOAc, ethyl acetate extract; aqu, aqueous extract.

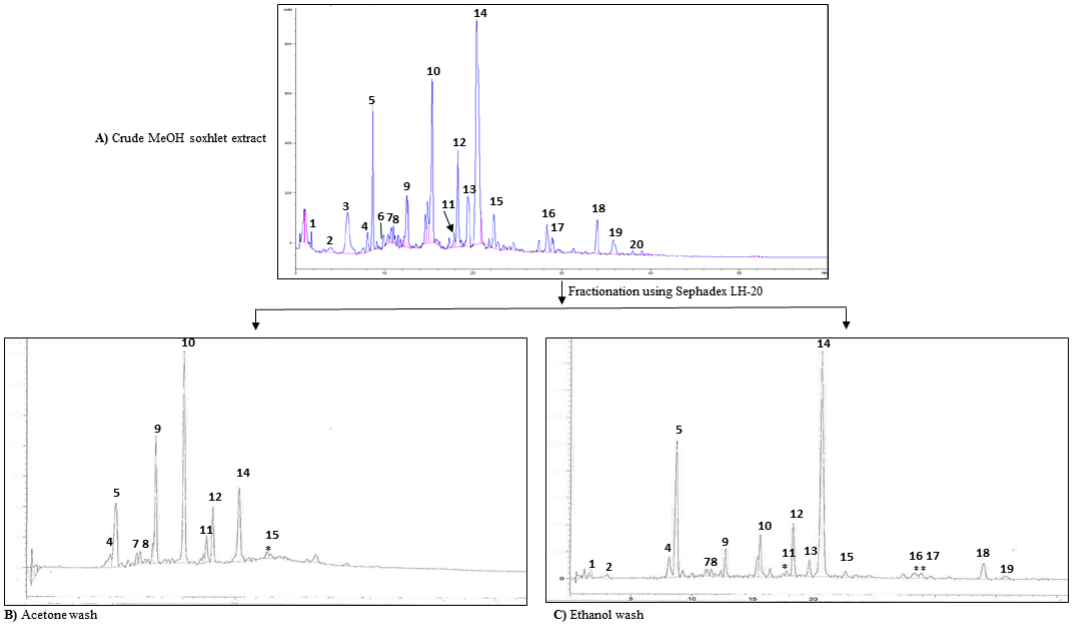


Fig. 4. Crude MeOH Soxhlet extract of the root of *Terminalia brownii* (A) and its Sephadex-LH 20 fractions (B and C). Gallic acid **(1)**; unknown ellagitannin [M-H]$^-$ 456.9961 **(2)**; unknown ellagitannin **(3)**; unknown ellagitannin **(4)** and isomer of methyl-S-flavogallonate **(5)**; gallotannin **(6)**; ellagitannin **(7)**; ellagitannin **(8)**; methyl-(*S*)-flavogallonate **(9)**; ellagitannin [M-H]$^-$ 609.1088 **(10)**; ellagic acid glucuronide **(11)**; ellagic acid xyloside **(12)**; methyl ellagic acid xyloside **(13)**; ellagic acid **(14)**; trimethyl ellagic acid **(15)**; ellagitannin [M-H]$^-$ 725.4141 **(16)**; ellagitannin [M-H]$^-$ 817.4003 **(17)**; acetylated ellagic acid derivative **(18)**; two ellagitannins at [M-H]$^-$ 577.1369 and 577.1392 **(19)**; ellagitannin [M-H]$^-$ 817.3999 **(20)**.

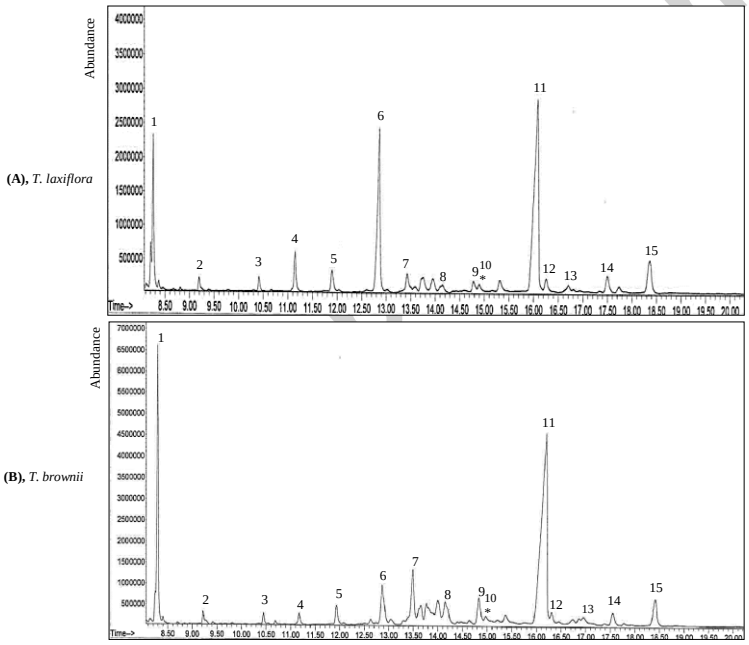


Fig. 5. GC/MS chromatogram of fatty acids and triterpenes in a hexane extract of the stem bark of (A) *T. laxiflora* and (B) *T. brownii*. TMS-1,18-octadec-9-ene dioate **(1)**;TMS-tetracosanoic acid **(2)**; TMS-hexacosanoic acid **(3)**; TMS-octacosanol **(4)**; TMS-octacosanoic acid **(5)**; TMS-triacontanol **(6)**; TMS- β-sitosterol **(7)**; TMS-β-amyrine **(8)**; stigmast-4-en-3-one **(9)**; TMS-dotriacontanol **(10)**; TMS-friedelin **(11)**; TMS-betulinic acid **(12)**; 5α-stigmastan-3,6-dione **(13)**; TMS-oleanane-type triterpenoids **(14)** and **(15).**

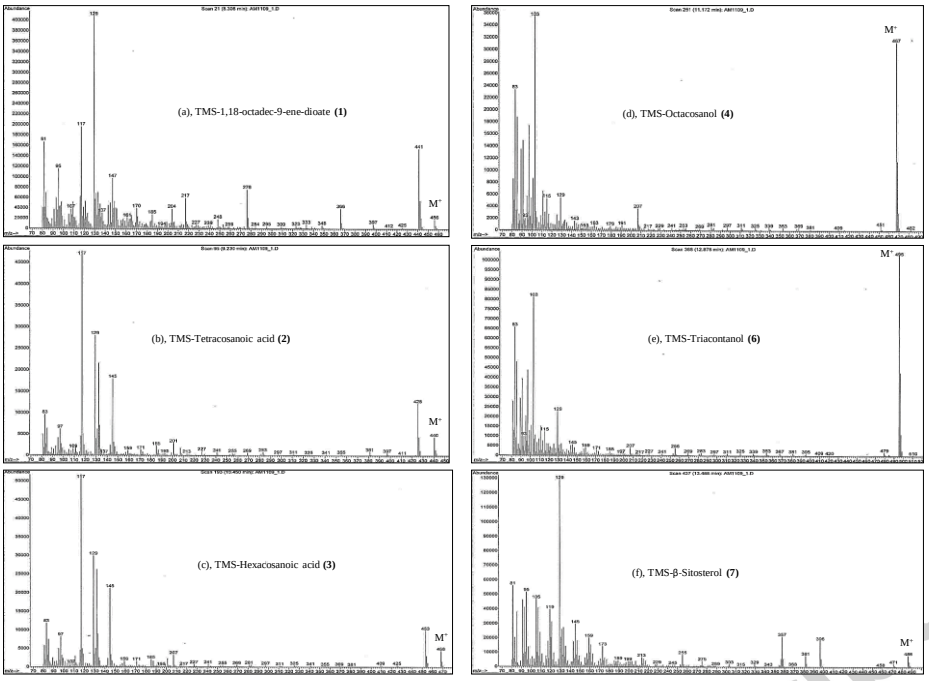
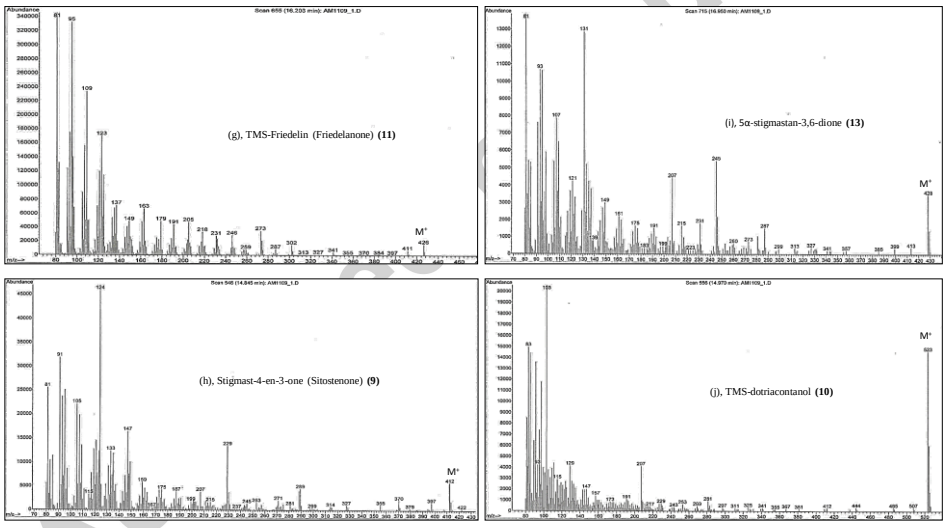


Fig. 6 a-f. TMS mass spectra of compounds (**1), (2), (3), (4), (6) and (7)** in *T. brownii* and *T. laxiflora* (numbering according to Fig. 5 and Table 5).



Continued Fig. 6 g-j. TMS mass spectra for compounds (**9), (10),** (**11)** and (**13).**