Tannins, flavonoids and stilbenes in extracts of African savanna woodland trees *Terminalia brownii*, *Terminalia laxiflora* and *Anogeissus leiocarpus* showing promising antibacterial potential

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Abstract

Terminalia laxiflora, *Terminalia brownii* and *Anogeissus leiocarpus* are used as decoctions, macerations, infusions and fumigations in East and West African traditional medicine for treatment of infectious diseases and their symptoms. Using this ethnopharmacological information as a guideline for our research and owing to the fact that these species have not been subjected to in depth antibacterial and phytochemical studies, thirty-nine extracts of various polarities of the stem bark, stem wood and roots were studied for growth inhibitory effects against the human pathogenic bacteria *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Micrococcus luteus* and *Pseudomonas aeruginosa*. Our results indicate that the studied species contain antibacterial compounds of a wide range of polarities. All polar root extracts of *T. laxiflora* and various polar extracts of *T. brownii* roots, including hot water decoctions, gave broad-spectrum antibacterial effects and low MIC values of 39 µg/ml. The main ellagitannins in an ethyl acetate extract of the root of *T. laxiflora* were found to be corilagin and its derivative and punicalagin. A methanol extract of the roots of *T. brownii* contained methyl-(S)-flavogallonate and its derivative as the main identified ellagitannins. Moreover, both *Terminalia* species were found to contain ellagic acid xylopyranoside and methyl ellagic acid xyloside and pure ellagic acid was present in *T. brownii*. Pure punicalagin did not give as low MIC as an ethyl acetate extract of the roots of *T. laxiflora*, containing punicalagin as one of its main compounds, although this ellagitannin totally inhibited the growth of *S. aureus* at 125 µg/ml and *P. aeruginosa* at 500 µg/ml. Similarly, pure ellagic and gallic acid gave higher MIC values than the methanolic root extract of *T. brownii* against *S. aureus* and *P. aeruginosa*. Moreover, a Sephadex LH-20 fraction of the methanolic extract of the roots of *T. brownii*, enriched with methyl-(S)-flavogallonate and its isomer, gave higher MIC values than the crude methanolic extract. These results suggest that the polyphenols in the extracts might act synergistically with each other. A methanolic soxhlet extract of the roots of *A. leiocarpus*, containing ampelopsin, aromadendrin, taxifolin, pinosylvin and 4'-

methylpinosylvin gave a low MIC value of 39 µg/ml against all bacterial strains used in this investigation. Our results demonstrate that the roots, stem bark and stem wood of *T. brownii*, *T. laxiflora* and *A. leiocarpus* are rich sources of (new) antimicrobial compounds and justify the uses of these plants for treatment of infections in African traditional medicine.

Keywords: *Terminalia laxiflora*; *Terminalia brownii*; *Anogeissus leiocarpus*; antibacterial; Africa; polyphenols

Abbreviations: IZ, inhibition zone; CFU/ml, colony forming units/ml

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1. Introduction

Bacterial infections such as acute respiratory disorders and diarrhoea are still the number one cause of death among children aged under five in Sub-Saharan Africa (Mulholland and Adegbola, 2005) and in most developing countries globally (WHO, 2003). It has been estimated that nearly half of all deaths in tropical countries are caused by infectious diseases (Okigbo and Ajalie, 2005). Antibiotic resistance has become a growing problem in Africa as in the rest of the world (Spencer, 2007). In Africa, this resistance is aggravated by the HIV epidemic, malnutrition and inadequate antibiotic treatments. In the developing world, including Africa, few suitable and affordable conventional antibiotic therapies are available (WHO, 2003).

Africa is a rich source of medicinal plants and depending on the country an estimated 60-80 % of the populations are using plants as readily available and affordable phytomedicines (Apulu et al., 1994; Mahomoodally, 2013; Schmelzer and Gurib-Fakim, 2013). According to the World Health Assembly (WHA) more evidence-based research is needed to elucidate the active compounds in traditional phytomedicines as well as to reveal and eliminate toxic compounds. New antimicrobial compounds with new mechanisms of actions could be found from these phytomedicines and from plant species based on their ethnomedical use for treatment of infections (Cos et al., 2006).

Phytomedicines and compounds isolated from them could be used in combination with conventional antibiotics to enhance their effects as well as to counteract multi-drug resistance (MDR) (Rasoanaivo et al., 2011).

The genus *Terminalia* (Combretaceae) is broadly distributed over the world in deciduous woodland and bushy grassland habitats and comprises 200 tropical and subtropical species (Foyet & Nana, 2013; Wickens, 1973). Thirty species of *Terminalia* are native to Africa (Wickens, 1973). Most of these species of *Terminalia* have some applications in African traditional medicine and many of them are used for the treatment of infectious diseases (Neuwinger, 2000). Species of *Terminalia* are a rich source of polyphenols, and among them ellagitannins (Avula et al., 2013; Juang et al., 2004; Tanaka et al., 1986). These ellagitannins are extracted in hot water decoctions used in traditional medicine and might be responsible for many of their beneficial health effects, including antibacterial properties. Polyphenolic compounds, and especially ellagitannins in African *Terminalia* species have been characterized to a rather limited extent, however (Pfundstein et al., 2010).

Terminalia laxiflora Engl. & Diels and *T. brownii* Fresen are used traditionally as decoctions, macerations, infusions, ointments, liniments and fumigations for treating a myriad of conditions and diseases, including bacterial and fungal infections, cough, diarrhoea, yellow fever, wounds, skin and venereal diseases (Table 1). The various ways in which these two species are made into traditional medicinal preparations indicate that they contain a range of antimicrobial compounds belonging to different chemical classes. Despite the various uses in traditional medicine just a few studies have been conducted on the antimicrobial activities and phytochemistry of extracts of these species. Extracts of *T. brownii* were found to give promising growth inhibitory effects against sweet potato pathogens (Opiyo et al., 2011) and human pathogenic bacteria (Mbwambo et al., 2007) as well as antimycoplasmal effects (Muraina et al., 2010). Data on characterization of compounds from *T. brownii* is scarce: triterpenoids and anti-*Pseudomonas* ellagic acid derivatives were reported recently in the stem bark of *T. brownii* (Machumi et al., 2013). In addition, the stem bark of *T. brownii* has been reported to contain a chromone derivative, terminalianone as well as the ellagitannins punicalagin and terchebulin (Negishi et al., 2011; Yamauchi et al., 2016). Recently, stem wood extracts of *T. laxiflora* were demonstrated to give promising *in vitro* anti-acne effects (Muddathir and Mitsunga, 2013) and leaf extracts were found to give antimycoplasmal effects (Muraina et al., 2010). Moreover, extracts of the root bark of *T. laxiflora* were found to give good antibacterial effects (Fasola et al., 2013). To our knowledge only a couple of papers report on the phytochemical composition of *T. laxiflora*; Laxiflorine, a polyhydroxy-lactone, has been reported from the root bark and some ellagic acid derivatives from the heartwood (Ekong and Idemudia, 1967). The ellagitannins,

flavogallonic acid dilactone and terchebulin as well as ellagic acid were found to be present in stem wood extracts (Muddathir et al., 2013).

The genus *Anogeissus* comprises fourteen species in Africa (Rogers and Verotta, 1996; Eloff, 1999). In Sudan the genus is represented by one species, *Anogeissus leiocarpus* (DC.) Guill. & Perr, [previously known as *Conocarpus leiocarpus* DC., Prodr. (1828) and *Anogeissus schimperi* Hochst. ex Hutch. & Dalziel (1927)] (El Amin, 1990; El Ghazali et al., 2003). *A. leiocarpus*, also known as the chewing stick or axlewood tree (Ndjonka et al., 2014; Salau et al., 2013), has a long history of traditional uses in Africa for treatment of a broad range of ailments. Roots, leaves, stem bark and twigs are used for treatment of gonorrhoea, cough, wounds, acute respiratory tract infections, stomach infections, fever and tuberculosis as well as parasitic diseases, among other uses (Table 1). The Yoruba people in Nigeria use *A. leiocarpus* to treat bacterial infections in general, and both roots and twigs are used as chewing sticks for dental hygiene (Dweek, 1996). *A. leiocarpus* is used externally as decoctions and fumigations for treatment of skin diseases and internally as hot water decoctions and infusions for the treatment of cough (Dweek, 1996). In agreement with these traditional uses, the extracts of the stem bark, roots, fruits and leaves of *A. leiocarpus* have been found to exert growth inhibitory effects against various human pathogenic bacteria, yeasts and fungi (Akande and Hayashi, 1998; Batawila et al., 2005; Elegami et al., 2002; Koné and Atindehou, 2008; Kubmarawa et al., 2007; Mann et al., 2008 a, b, c; Mann et al., 2010; Mann, 2012; Ogundiya et al., 2006; Rajpoot and Singh, 2014; Sanogo et al., 1998; Taiwo et al., 1999). Flavonoids, ellagitannins, ellagic acid derivatives, triterpenoids and alkaloids are known from *A. leiocarpus* (Attioua et al., 2011; Hubert et al., 2014; Kubmarawa et al., 2007; Ndjonka et al., 2014; Rajpoot and Singh, 2014; Shuaibu et al., 2008). The roots of *A. leiocarpus* have, however, to the best of our knowledge not been investigated in detail for their ellagitannins, flavonoids and other compounds, although Salau et al. (2013) have made a general screening of compound classes in the root bark, and reported the presence of tannins, flavonoids, alkaloids, saponins, phlobatannins and terpenes. To date three ellagitannins, castalagin, punicalagin and terchebulin are known from the stem bark of *A. leiocarpus* (Shuaibu et al., 2008).

In this investigation, using an ethnomedicinal approach and in an attempt to find extracts with good antibacterial potential, thirty-nine extracts of various polarities of *T. brownii*, *T. laxiflora* and *A. leiocarpus* roots, stem bark and stem wood were screened for antibacterial effects against *Staphylococcus aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *Micrococcus luteus* ATCC 4698 and *Pseudomonas aeruginosa* ATCC 27853. Our study focused especially on the antibacterial effects and chemical composition of the roots of the studied plants, since to the best of our knowledge, there exists only a few studies in this respect. Likewise, *A. leiocarpus* stem wood and *T. laxiflora* root extracts have not been screened against *M. luteus*, *S. aureus* and *S. epidermidis*. Some of the extracts

were prepared as hot water decoctions to imitate traditional medicinal preparations. Extracts giving especially promising antibacterial effects, such as an ethyl acetate extract of the stem bark of *A. leiocarpus*, methanolic soxhlet root extracts of *A. leiocarpus* and *T. brownii* as well as an ethyl acetate extract of *T. laxiflora* roots were selected for HPLC-DAD and UHPLC/Q-TOF MS analysis with emphasis on their ellagitannin and flavonoid composition. For the first time, $[M-H]^-$ data, UV λmax and HPLC retention times of various phenolic compounds as well as HPLC fingerprints of *T. brownii*, *T. laxiflora* and *A. leiocarpus* extracts are presented.

2. Materials and Methods

2.1. Plant material collection

Stem wood, stem bark and roots of the plants used in this study were collected in May to June 2006 and in August 2012 from El Nour Natural Forest Reserve, southeast of Ed Damazin, Sudan (Table 1). The plants were collected by the first author and Mr. Gibreel H. (PhD) and identified at the Faculty of Forestry, University of Khartoum, Sudan. Voucher specimens are deposited at the Faculty of Forestry and Faculty of Sciences, University of Khartoum, Sudan.

2.2. Soxhlet extraction, sequential extraction and solvent partition

Samples of roots and stem logs of the diameter 20-42 cm from healthy trees were collected. Root, stem wood and stem bark were dried and cut into small chips using a sawing machine followed by a hammer mill (mesh.) to obtain finely ground samples.

Soxhlet extraction was performed in order to obtain crude methanolic extracts. 20 g of plant powder was added to a soxhlet sock and 500 ml of methanol (MeOH) was poured into a soxhlet flask. The soxhlet extraction continued for five hours whereafter the extract was dried in vacuo in a Rotavapor machine (Heidolph VV2000) and freeze-dried in a lyophilizer for two days.

Sequential extraction was performed using 100 g of the powdered dry plant material and solvents of increasing polarities. Initially, the plant material was extracted using 1600 ml petroleum ether (PE), followed by extraction with 1400 ml chloroform ($CHCl_3$). The residue was then extracted with 80 % methanol for all other plant parts except for the root part for which marc was extracted with acetone before adding 80 % methanol. The 80 % methanol extract was further fractionated by solvent partition using ethyl acetate (EtOAc) in order to obtain ethyl acetate and aqueous fractions. The filtered extracts were concentrated to dryness in vacuo using a Rotavapor machine (Heidolph VV2000) and then freeze-dried for two days in a lyophilizer.

Decoctions were made according to a procedure used by traditional healers in Sudan: 20 g plant powder was mixed with water (500 ml) which was brought to boil. The decoction was centrifuged at 2000 rpm for 10-15 minutes (Eppendorf AG centrifuge 5810R, Germany) and filtered using filter paper (Schleicher & Schuell, ∅ 150 mm, Germany) whereafter it was freeze-dried for two days.

All the freeze-dried extracts were dissolved in methanol to give a final concentration of 50 mg/ml. These stock solutions were used for initial antibacterial screening using an agar disk diffusion method.

2.3. Sephadex LH-20 chromatography

A root methanolic soxhlet extract of *Terminalia brownii* was subjected to Sephadex LH-20 (Pharmacia, Uppsala, Sweden) fractionation according to Ann Hagerman, Tannin handbook (Hagerman, 2011). 200 mg dry root methanolic soxhlet extract was dissolved in 10 ml of 80% (v/v) EtOH. The extract was centrifuged whereafter it was poured into a 50 ml volume plastic centrifuge tube (Eppendorf) containing 2.5 g Sephadex LH-20. 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 colourless and showed an absorbance close to zero at 280 nm. The supernatant (EtOH wash) was collected into a rotary evaporator flask. The tannin fraction (acetone wash) was released from the Sephadex LH-20 using 2 × 15 ml 70% acetone. The acetone wash was filtrated using double filter paper into a 100 ml rotary evaporation flask to be and concentrated to dryness using a rotary evaporator. The 80 % EtOH wash was also reduced to dryness using a rotary evaporator. The acetone and EtOH washes were dissolved in 5 ml H_2O before dried using liquid nitrogen, whereafter they were freeze-dried for two days. The freeze-dried fractions were dissolved in MeOH to make stock solutions of 30 mg/ml for antibacterial testing.

Table 1

Summary of traditional uses of *Terminalia laxiflora, T. brownii and Anogeissus leiocarpus* collected in Sudan in 2006 and 2012. Vernacular names according to arabic language and local dialects in Sudan. Voucher numbers refer to deposits at University of Khartoum, Sudan.

Plant botanical name	Voucher No.	Vernacular name	Plant parts used	Traditional uses	References
Terminalia laxiflora Engl. & Diels.	Plate 24. Jun, 2006.	Darout or Sufaraya	stem, root, leaves, fruit	malaria, cough, bronchitis, back pains, venereal inflammations, bacterial infections, fever, skin pustules, wounds and hemostatic hemorrhoids, rheumatism, eye diseases, anti-inflammatory, tonic and skin diseases, diarrhoea, dysentery, jaundice, diuretic.	Doka and Yagi, 2009; El Ghazali et al., 1987; El Ghazali et al., 1997; El Ghazali et al., 2003; Fasola et al., 2013; Foyet & Nana 2013; Haxaire, 1979; Koné and Atindehou, 2008; Muddathir et al., 2013; Musa et al., 2011; Neuwinger, 2000; Salih, personal communication.
Terminalia brownii Fresen	Plate 23. Jun, 2006.	Subagh or Alshaf	stem, leaves, root	malaria, cough, bronchitis, back pain, venereal diseases, bacterial infections, fever, skin pustules, wounds and hemostatic hemorrhoids, allergy, gastric ulcers, stomach ache, rheumatism, diarrhoea, anti-inflammatory, tonic, chest coughs, skin diseases, diabetes, allergy, family planning, worms, kidney disorders, yellow fever.	Abd alla et al., 2013; Doka and Yagi, 2009; El Ghazali et al., 1997; El Ghazali et al., 2003; Kareru et al., 2007, 2008; Keter and Mutiso, 2012; Machumi et al., 2013; Mariod et al., 2014; Mbwambo et al., 2007; Mosango, 2013; Musa et al., 2011; Muthee et al., 2011; Neuwinger, 2000. Salih, personal communication.
Anogeissus leiocarpus (DC.) Guill. & Perr.	Plate 16. Jun, 2006.	Sahab or Seilak	stem, leaves, root, fruit	Anti-inflammatory, dysentery, cough, tuberculosis, giardiasis, malaria, trypanosomiasis, yellow fever, vermifuge, jaundice, pathogenic microbial infections, gonorrhea, wounds.	Akanbi et al., 2014; Batawila et al., 2005; Elegami et al., 2002; El Ghazali et al., 2003; Mann et al., 2008 a, b, c; Mann, 2012; Mann et al., 2014; Muraina et al., 2010; Musa et al., 2011; Ndjonka et al., 2012; Ndjonka et al., 2013. Okpekon et al., 2004; Taiwo et al., 1999; Rajpooth & Singh, 2014; Gbadamosi & Ogunsuyi, 2014; Salih, personal communication.

2.4. High performance liquid chromatography (HPLC-UV/DAD) and mass spectrometry (UHPLC/ Q-TOF MS)

2.4.1. HPLC-UV/DAD method

HPLC analysis was performed using a method described by Julkunen-Tiitto and Sorsa (2001), developed for detection of polyphenols. The liquid chromatographic system consisted of a Waters 600 E pump and a controller coupled to a 991 PDA detector. An autosampler controlled by Agilent Chemstation software (Water Corp., Milford, USA) was used. Separations were performed on a reversed phase Hypersil Rp C_{18} column (length: 10 mm; ID: 2 mm). 10 µl of samples (2 mg/ml in 50% MeOH) were injected. Gradient elution was performed at a flow rate of 2 ml/min using two solvent systems: A) Aqueous 1.5% tetrahydrofuran + 0.25% orthophosphoric acid and B) 100 % MeOH. UV fingerprint chromatograms were constructed at 220, 270, 280, 320 and 360 nm. UVλ absorption maxima spectra of the compounds of interest were recorded between 200 and 400 nm using Agilent Chemstation software. Compounds of interest were compared to commercial compounds and literature (Conrad et al., 2001; Pfundstein et al., 2010).

2.4.2. UHPLC/ Q-TOF MS method

The compounds were identified using UHPLC-DAD (Model 1200 Agilent Technologies)-JETSTREAM/QTOFMS (Model 6340 Agilent Technologies) equipped with a 2.1 x 60 mm, 1.7 µm C_{18} column (Agilent technologies) as described in Taulavuori et al. (2013). Shortly, the solvent A was 1.5% tetrahydrofuran and 0.25% acetic acid in HPLC quality water and the solvent B was 100% methanol. A gradient run was 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%, and from 20 to 22 min, 100 to 0% B. The mass spectra were acquired at positive and negative ion mode depending on the compounds and the mass range was from 100 to 2000 m/z.

2.5. Bacterial strains and assays used for screening antibacterial activity

2.5.1. Bacterial strains

The growth inhibitory activity of the stem wood, stem bark and root extracts of *Terminalia laxiflora, Anogeissus leiocarpus* and *Terminalia brownii* were investigated against four bacterial strains. Three Gram-positive and one Gram-negative strain were used; *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Micrococcus luteus* ATCC 4698 and *Pseudomonas*

aeruginosa ATCC 27853. All strains were obtained from the Faculty of Pharmacy, Division of Pharmaceutical Biosciences, University of Helsinki, Finland.

2.5.2. Standard antibiotics and pure compounds

Penicillin-G (Fluka, Germany), gentamycin (Sigma-Aldrich, St. Louis MO, USA), tetracycline hydrochloride (Sigma-Aldrich, St. Louis MO, USA) and ampicillin (Sigma-Aldrich, St. Louis MO, USA) were used as standard antibiotics. Punicalagin (CAS 65995-63-3 from pomegranate, Sigma-Aldrich, Germany), ellagic acid (E-2250 from chestnut bark, Sigma-Aldrich, England) and gallic acid (G-7384, Sigma-Aldrich, China) were used as pure compounds.

2.5.3. Agar disk diffusion method

Extracts of the roots, stem bark and stem wood of *Terminalia laxiflora, Anogeissus leiocarpus* and *Terminalia brownii* were screened for their antibacterial effects using an agar disk diffusion method for initial screening (Fyhrquist et al., 2002; Rauha et al., 2000). Extracts and antibiotics were dissolved in methanol to obtain stock solutions of 50 and 10 mg/ml, respectively. Tetracycline, penicillin-G, ampicillin and gentamicin were used as positive controls.

Twenty-five ml of sterile base agar (Difco, VWR Finland) as bottom layer and twenty-five ml of isosensitest agar (OXOID, Thermo Fisher Scientific), as top layer were applied into sterile petri dishes (∅ = 14 cm, VWR Finland). To initiate a test, nutrient agar slants were inoculated with bacteria and grown overnight at 37˚C. Actively growing bacterial culture from the test tube was transferred into isotonic sodium chloride, whereafter the absorbance of the bacterial suspension was measured spectrophotometrically at 625 nm (UV-Visible Spectrophotometer, Pharmacia LKB-Biochrom 4060). Suspensions were diluted with 0.9 % (w/v) sodium chloride to an absorbance of 0.1 at 625 nm, corresponding to approximately 1×10^8 CFU/ ml. 200 µl of this suspension was spread evenly on each petri dish. Whatman filter paper discs (∅ = 12.7 mm, Scleicher & Shuell) containing 200 µl of 50 mg/ml extracts and 10 mg/ml antibiotics, respectively, were placed equidistantly on the agar surface. 200 µl methanol was pipetted on some filter papers as solvent control. The plates were incubated at +4˚C for 1 h prior to incubation overnight at +37˚C. The diameter of inhibition zones were measured and expressed as the mean of four diameters ± SEM.

For extracts which builded up excess precipitation, reliable minimum inhibitory concentration (MIC) could not be estimated using the turbidimetric microdilution method and therefore approximate MIC values were determined using agar diffusion. For MIC estimations, 200 µl of two-fold dilutions of the extracts and antibiotics from 5 mg/ml to 39 µg/ml and from 1 mg/ml to 0.0076 µg/ml,

respectively, were pipetted onto Whatman filter papers (∅ = 12.7 mm). The approximate MIC was estimated as the lowest mean inhibitory concentration of three tests (triplicates) ± SEM that gave a clear zone of inhibition (radius of IZ measured from the edge of the filter paper disc was not more than 1 mm) around the filterpaper disc after incubation at +37˚C for 24 h.

2.5.4. Microdilution method

A microdilution turbidimetric broth method based on the guidelines of Clinical and Laboratory Standards Institute (2012) was used for determination of MIC (Minimum inhibitory concentration) of the most promising extracts selected from agar diffusion screening results. Extracts (50 mg/ml) were dissolved in methanol and two-fold serially diluted in Mueller Hinton Broth (Becton, Dickinson & Company, USA) in sterile Eppendorf tubes from 5 mg/ml to 19.53 µg/ml. A Sephadex LH-20 ethanol fraction of *T. brownii* roots was two-fold serially diluted from 3 mg/ml (in 10% v/v MeOH) to 11.72 µg/ml using Mueller Hinton broth. Ellagic acid, gallic acid and punicalagin were prepared as 1 mg/ml stock solutions in 10% (v/v) dimethyl sulfoxide (DMSO, Sigma-Aldrich, France) and two-fold serially diluted with broth until a concentration of 31.25 µg/ml was reached. Tetracycline, penicillin, ampicillin and gentamicin were used as positive controls from 1 mg/ml to 0.0076 µg/ml. 100 µl of the dilutions were pipetted onto a 96 well microtitre plate (Nunc, Nunclone, Denmark). Methanol and DMSO were used as solvent controls, and were found not to affect the growth of the bacteria at the concentrations used in the tests (5 % v/v or less). All bacterial cultures were grown overnight either in Mueller Hinton broth or on Nutrient agar slants at +37˚C before the test. 1 ml of Mueller Hinton bacterial suspension or inoculum transferred from nutrient agar slant to 0.9% (w/v) sodium chloride were measured for turbidity at 625 nm using a UV-Visible Spectrophotometer type 1510 (Thermo Fisher Scientific Oy) and were diluted to an absorbance of 0.1 (approximately 1.0×10^8 CFU/ ml). The bacterial suspensions were diluted further so that the final number of cells in the inoculum was 1.0×10^6 CFU/ml. A portion of 100 µl of this inoculum was added to the wells of the microplate (Nunc, Nunclone, Denmark) together with 100 µl extracts, fractions, pure compounds or antibiotics, the wells thus containing 5×10^5 CFU/ml. The microplates were incubated for 24 h at +37˚C, whereafter the turbidity of the wells at 620 nm was recorded using Victor 1420 (Wallac, Finland) spectrophotometer. All assays were done in quadruplicate and the % inhibition of growth results were expressed as mean ± standard error of mean (SEM). The minimum inhibitory concentration (MIC) was estimated as the lowest concentration that inhibited 90 % or more of the growth of the studied bacterial strains (growth controls).

2.6. Statistical analysis of data

All data (diameters of inhibition and percentage inhibition of growth) were expressed as mean ± SEM, obtained from three-four independent experiments, each performed in duplicate, triplicate or quadruplicate.

3. Results and discussion

3.1. Antibacterial results

Thirty-nine extracts of various polarities, including decoctions of the roots, stem bark and stem wood of the traditional medicinal plant species *Terminalia laxiflora*, *T. brownii* and *Anogeissus leiocarpus* were screened for their antibacterial effects (Table 2 and 3). The plant species were chosen according to their reported uses for treatment of bacterial infections in African traditional medicine (Table 1) and due to the fact that these species only to a limited extent have been investigated for their antimicrobial effects and compounds especially when it comes to the root part. Clinically relevant human pathogenic bacteria were used for the screenings. Of these bacteria, especially *Staphylococcus aureus* and *Pseudomonas aeruginosa* are known for their ability to develop antibiotic resistance. Suitable treatment regimens against multi-drug resistant *S. aureus* have been narrowed to just a few agents, among them the aminoglycoside vancomycin (Appelbaum, 2006; Mwangi et al., 2007). *P. aeruginosa* has been reported to develop resistance to multiple classes of antibiotics (Poole, 2011) and easily develops antibiotic resistance due to several efflux pump systems in the membranes (Li et al., 1994) as well as due to its ability to form biofilms during pathogenesis (Rasamiravaka et al., 2014). Our results demonstrate that *T. laxiflora*, *T. brownii* and *A. leiocarpus* contain compounds that strongly reduce the growth of *S. aureus* and *P. aeruginosa*, and that some extracts were more effective growth inhibitors than conventional antibiotics.

3.1.1. Terminalia laxiflora

We report for the first time that extracts of the root, stem bark and stem wood of *T. laxiflora* give promising antibacterial effects against *S. aureus, S. epidermidis* and *M. luteus* (Tables 2 and 3).

Root extracts

The most outstanding growth inhibitory effects in terms of minimum inhibitory concentration were obtained by all the investigated polar root extracts of *T. laxiflora*, giving low MIC values of 39.06 µg/ml against all bacterial strains in this screening (Table 3). We found that our MIC values

correlated well with our agar diffusion results, as all root extracts giving low MIC values also demonstrated large inhibition zones (Table 2). Interestingly, a hot water decoction made according to guidelines of traditional healers in Africa gave promising growth inhibitory effects against all bacterial species (IZ: 24-39 mm, MIC 39.06 – 78.12 µg/ml) (Table 2) and the effects were dose-dependent against *M. luteus* (Fig. 6). Our results are in agreement with one previous investigation in which root bark extracts of *T. laxiflora* were reported to show good antibacterial effects against *P. aeruginosa* (Fasola et al., 2013). Fasola et al. (2013) did not include *S. aureus* and *S. epidermidis* in their investigation and therefore our results now indicate that the root of *T. laxiflora* contains compounds active against Staphylococci as well. In summary our results on the good antibacterial effects of *T. laxiflora* root extracts and decoction support the traditional medicinal uses of the roots and root bark of this plant for diarrhoea and wounds (Fasola et al., 2013; Foyet & Nana, 2013) since *P. aeruginosa* and *S. aureus* are often present in chronic wounds (Serra et al., 2015) and *S. aureus* can be a causative agent of diarrhoea (Kadariya et al., 2014).

We hypothesize that the ellagic acid derivatives, punicalagin and gallic acid present in the ethyl acetate extract of *T. laxiflora* roots (Table 4) contribute to its significant antibacterial effects, since related compounds from the root of *T. macroptera* have confirmed efficiency against clininal strains of Gram-negative bacteria (Silva et al., 2012). Notably, we found that the ethyl acetate extract of the roots of *T. laxiflora* was a more effective growth inhibitor than tetracycline and gentamycine against *P. aeruginosa* (Table 3), and this activity is suggested to be due partly to punicalagin which we found in high concentrations in this extract (12.9 % peak area, Table 4). Reddy et al. (2007) found that punicalagin inhibited the growth of *P. aeruginosa* at a very low IC50 concentration of 3.2 µM (equal to 1.8 µg/ml). In contrast to their results, however, we found that the MIC value of punicalagin was 125 µg/ml against *S. aureus* and 500 µg/ml against *P. aeruginosa*. Moreover, these MIC values were significantly higher than our MIC of 39 µg/ml for the crude ethyl acetate extract (Table 3). This might be due to synergistic effects between the compounds in the extract, or due to some unkown and very powerful antibacterial compound present in the extract. Our results on MIC values of punicalagin are well in accordance with the results of Burapadaja & Bunchoo (1995) and Taguri et al. (2006).

Table 2
Antimicrobial activities of extracts of stem wood, stem bark and roots of *Terminalia laxiflora*, *T. brownii* and *Anogeissus leiocarpus*. Results obtained with an agar diffusion method.

Extracts and antibiotics	***M. luteus***	***S. aureus***	***S. epidermidis***	***P. aeruginosa***
***T. laxiflora* (S.W.):**				
Pt	NT	NT	**23 ± 0.29**	NT
$CHCl_3$	**21 ± 0.10**	18 ± 0.33	18 ± 0.17	**32 ± 0.00**
EtOAc	**26 ± 0.20**	**23 ± 0.41**	**22 ± 0.50**	**21 ± 0.50**
Aqueous	**25 ± 0.10**	**21 ± 0.41**	**20 ± 0.33**	19 ± 0.44
MeSox	**26 ± 0.33**	19 ± 0.00	18 ± 0.33	17 ± 0.33
***T. laxiflora* (S.B.):**				
Pt	19 ± 0.10	NT	**22 ± 0.33**	17 ± 0.17
$CHCl_3$	**23 ± 0.00**	16 ± 0.44	**21 ± 0.17**	17 ± 0.17
EtOAc	**27 ± 0.33**	**20 ± 0.30**	**20 ± 0.33**	**21 ± 0.33**
Aqueous	19 ± 0.10	15 ± 0.00	**20 ± 0.33**	18 ± 0.10
MeSox	**22 ± 0.33**	**21 ± 0.33**	19 ± 0.33	**23 ± 0.33**
***T. laxiflora* (R):**				
HH2O	**39 ± 0.33**	**25 ± 0.33**	**24 ± 0.17**	**25 ± 0.29**
MeSox	**39 ± 0.29**	**22 ± 0.00**	**22 ± 0.00**	**25 ± 0.17**
acetone	**36 ± 0.17**	**22 ± 0.33**	**22 ± 0.17**	**21 ± 0.17**
EtOAc	**37 ± 0.00**	**21 ± 0.17**	**20 ± 0.17**	**22 ± 0.17**
***T. brownii* (S.W.):**				
$CHCl_3$	18 ± 0.30	19 ± 0.16	NT	18 ± 0.29
EtOAc	**24 ± 0.30**	**20 ± 0.16**	17 ± 0.29	18 ± 0.29
MeSox	**26 ± 0.33**	**20 ± 0.30**	19 ± 0.00	16 ± 0.33
***T. brownii* (S.B.):**				
$CHCl_3$	**20 ± 0.03**	18 ± 0.44	**21 ± 0.17**	16 ± 0.29
EtOAc	**22 ± 0.40**	17 ± 0.33	**20 ± 0.17**	**26 ± 0.50**
Aqueous	19 ± 0.30	**20 ± 0.44**	**22 ± 0.50**	16 ± 0.29
MeSox	**24 ± 0.30**	**21 ± 0.30**	19 ± 0.20	19 ± 0.33
***T. brownii* (R):**				
HH2O	**37 ± 0.00**	**21 ± 0.17**	**20 ± 0.17**	**22 ± 0.17**
MeSox	**39 ± 0.17**	**22 ± 0.00**	**21 ± 0.17**	**22 ± 0.29**
acetone	**39 ± 0.00**	**23 ± 0.17**	**22 ± 0.00**	**20 ± 0.29**
EtOAc	**38 ± 0.00**	**20 ± 0.17**	**19 ± 0.00**	**20 ± 0.44**
***A. leiocarpus* (S.W.):**				
Pt	NT	NT	**30 ± 0.17**	15 ± 0.20
$CHCl_3$	16 ± 0.20	15 ± 0.50	18 ± 0.17	**28 ± 0.00**
EtOAc	NT	19 ± 0.33	NT	NT
Aqueous	**24 ± 0.10**	**21 ± 0.33**	**23 ± 0.29**	**21 ± 0.10**
MeSox	**25 ± 0.00**	**20 ± 0.30**	**20 ± 0.00**	**26 ± 0.33**

Table 2
Continued.

Extracts and antibiotics	*M. luteus*	*S. aureus*	*S. epidermidis*	*P. aeruginosa*
***A. leiocarpus* (S.B.)**				
Pt	NT	NT	NT	16 ± 0.03
$CHCl_3$	17 ± 0.20	17 ± 0.33	19 ± 0.17	**24 ± 0.29**
EtOAc	**28 ± 0.30**	19 ± 0.33	**23 ± 0.29**	**22 ± 0.22**
Aqueous	**26 ± 0.33**	19 ± 0.33	**24 ± 0.17**	**21 ± 0.44**
MeSox	**30 ± 0.33**	**24 ± 0.00**	**22 ± 0.30**	**25 ± 0.30**
***A. leiocarpus* (R):**				
HH2O	**32 ± 0.33**	16 ± 0.00	18 ± 0.17	**24 ± 0.17**
MeSox	**36 ± 0.17**	**23 ± 0.17**	**28 ± 0.29**	**24 ± 0.44**
acetone	**36 ± 0.33**	**22 ± 0.20**	**23 ± 0.29**	**25 ± 0.29**
EtOAc	**36 ± 0.00**	**22 ± 0.17**	**23 ± 0.00**	**24 ± 0.29**
Antibiotics:				
Ampicillin	68 ± 0.02	64 ± 0.00	58 ± 0.00	NT
Gentamicin	NT	31 ± 0.03	45 ± 0.03	28 ± 0.03
Tetracycline	53 ± 0.01	53 ± 0.02	67 ± 0.02	33 ± 0.01
Penicillin	65 ± 0.00	68 ± 0.01	NT	NT

Diameters of inhibition in mm. Filter paper size: 12.7 mm. Two-hundred microliters of extracts (50 mg/ml) and antibiotics (10 mg/ml) were applied on the filter papers. Pt: Petroleum ether extracts; $CHCl_3$: Chloroform extracts; EtOAc: ethyl acetate extracts; Aqueous: 80 % aqueous methanol; HH2O: hot water decoction; MeSox: methanolic soxhlet extract; S.W.: Stem wood; S.B.: stem bark; R: roots; NT: not tested. Results expressed as the mean ± SEM of quadruplicates. Good results indicated in bold font (Ø > 20 mm) and exceptionally good activity (Ø > 30 mm) underlined.

We found that corilagin and its isomer are present in high concentrations in the *T. laxiflora* ethyl acetate root extract (6.7 and 20.4 % peak area, respectively, Table 4). Corilagin has been found to reduce the MIC of β-lactams, such as oxallicin, in methicillin-resistant *S. aureus* (MRSA) by suppressing β lactamase production as well as reducing the production of penicillin binding protein 2´ (Shimizu et al., 2001; Shiota et al., 2004). Corilagin has also been shown to possess good growth inhibitory effects against *Helicobacter pylorii* (Funatogawa et al., 2004) and against *Staphylococcus aureus* (Burapadaja & Bunchoo, 1995). Therefore it is certain that corilagin and its isomer contribute to the antibacterial effects against *S. aureus* we have seen for the *T. laxiflora* root extract.

The growth inhibitory activity of ellagitannins and tannin-related compounds against microorganisms varies according to their chemical structure, and has mainly been associated to their protein binding capacity (Goel et al., 2005), but recently also more specific mechanisms have been suggested to be involved (Eloff et al., 2008). For example, ellagitannins have been thought to act as Lipid A agonists, mimicking LPS/Lipid A activity, and therefore there are prospects to redesign ET structure to function as a Lipid A antagonist by binding competitively to LPS and hindering release of TNF-α causing bacterial induced sepsis (Coca et al., 2009). Dimeric ellagitannin analogues have showed low toxicity, good bioavailability and promising antagonistic action and might therefore represent interesting leads for further development of anti-sepsis therapies (Feldman et al., 2002). This

opens prospects for finding new antimicrobial compounds among the highly variable ellagitannins in the largely unexplored African species of *Terminalia*, in which yet new structures of ellagitannins are to be discovered. Interestingly, we have found that the mentioned ellagitannins corilagin and its derivative as well as punicalagin are present in high concentrations also in hot water decoctions of the roots of *T. laxiflora*, thus supposedly being the principal antimicrobial agents in these traditional medicinal preparations and supporting their use for treatment of infections, and especially infections related to wounds, "itchy eyes" and diarrhoea (Foyet & Nana, 2013). Studies on antibiotic agent modifying effects of ellagitannins (and other compounds) from *T. laxiflora* in combinations with various antibiotics should be taken into consideration since combining conventional antibiotics with adjuvant therapy has been found to be an effective strategy for overcoming drug resistance in resistant bacteria and a strategy to reuse antibiotics against which bacteria have evolved resistance (Shiota et al., 2004).

Stem bark and wood extracts

We found that various stem bark extracts of *T. laxiflora* gave promising antibacterial effects (Table 2 and Table 3). The highest activity was recorded for an ethyl acetate extract which gave a MIC of 39.06 µg/ml against *P. aeruginosa* and was more efficient than gentamycin (MIC 62.5 µg/ml) and tetracycline (MIC 125 µg/ml) against this bacterial strain (Table 3). Moreover, a methanolic soxhlet extract gave a low MIC value of 78.12 µg/ml against all Gram-positive bacteria. These results illustrate the need to explore *T. laxiflora* stem bark ethyl acetate and methanolic soxhlet extracts for antimicrobial compounds.

In terms of MIC we found that stem wood extracts of *T. laxiflora* were in general slightly less antibacterial than the stem bark and root extracts, although good antibacterial effects were demonstrated by some extracts of the stem wood such as ethyl acetate and methanolic soxhlet extracts (Table 3). The growth inhibitory effects of an ethyl acetate extract of the stem wood was dose-dependent against *S. aureus* (Fig. 7). Muddathir et al. (2013) demonstrated that a methanolic stem wood extract of *T. laxiflora*, as well as terchebulin and gallic acid isolated from this extract, possess strong antibacterial effects against *Propionibacterium acnes* (Muddathir et al., 2013), but the growth inhibitory effects of *T. laxiflora* wood extracts have not been explored earlier against *S. aureus* and *P. aeruginosa.* In our analysis, gallic acid gave rather high MIC values against *S. aureus* and *P. aeruginosa*, 500 and 250 µg/ml, respectively (Table 3). These results are in accordance with Taguri et al., (2006). Therefore we suggest that the good antibacterial effects of the stem wood methanol and ethyl acetate extracts of *T. laxiflora* against *S. aureus* and *P. aeruginosa*, giving MIC values of 78 and

156 µg/ml, respectively, might be due to a combination effect of the ellagitannins and gallic acid in the extracts.

Interestingly, a chloroform extract of the stem wood gave strong activity against *P. aeruginosa* expressed by large inhibition zones (IZ: 32 mm) and a petroleum ether extract gave good effects against *S. epidermidis* (IZ: 23 mm) (Table 2). This indicates that *T. laxiflora* stem wood also contains more nonpolar antimicrobial compounds to be explored.

Our results support the uses of decoctions and other applications such as powders of *T. laxiflora* stem bark and wood for treatment of wounds, bacterial infections and their symptoms such as diarrhoea and cough in African traditional medicine (Batawila et al., 2005; Foyet & Nana, 2013; Haxaire, 1979; Musa et al., 2011; Neuwinger, 2000). Our research, together with other authors, shows that *T. laxiflora* contains a variety of antimicrobial compounds of different polarities which should be studied more in depth. There could be a possibility to find new antibiotic molecular structures from this plant.

3.1.2. Terminalia brownii

We found that most of the investigated extracts of different polarities of the roots, stem bark and stem wood of *T. brownii* were antibacterially active, although the extent of activity differed between organs and extracts (Table 2 and 3). In general the polar extracts were more active, but good activities were also expressed with a chloroform extract of the stem wood indicating that *T. brownii* contains a wide spectrum of antibacterial compounds (Table 2). The polar extracts gave dose-dependent growth inhibitory effects.

Root extracts

Methanolic soxhlet extracts of the roots of *T. brownii* gave the most promising antibacterial effects of all tested organs and extracts expressing MIC values of 39.06 µg/ml against all bacteria (Table 3) and inhibition zones of 39 mm against *M. luteus* (Table 2). This result is in accordance with Mbwambo et al. (2007) who also found that root extracts of this plant were more antibacterial than extracts of stem bark and wood. We found that a methanolic soxhlet extract and a hot water decoction of the roots demonstrated substantial activity against *P. aeruginosa* being more effective than gentamycin and tetracyclin (Table 3). This result is especially interesting since *P. aeruginosa* is known for its high intrinsical resistance against antibiotics and its resistance to multiple classes of antibiotics is increasing (Poole, 2011). In HIV-infected persons, *Pseudomonas* can cause severe pneumonia and often leads to a necrotizing infection responding poorly to antibiotic treatment (Roilides et al., 1992).

We found that the methanolic soxhlet extract of *T.brownii* roots is rich in ellagitannins, among them methyl-(S)-flavogallonate and its derivative, as well as various ellagic acid derivatives (Fig. 2, Table 4). The promising antibacterial effects expressed by this extract could therefore be due to ellagitannins. In an attempt to separate ellagitannin enriched fractions from this highly antibacterial extract, we performed Sephadex LH-20 fractionation. The resulting ethanol wash, enriched with methyl-S-flavogallonate and its isomer was obtained. This fraction, however, gave a higher MIC value than the crude extract against *S. aureus* and *P. aeruginosa* (Table 3). This result shows a trend similar to that of the pure phenols and polyphenols (gallic acid, ellagic acid and punicalagin) compared to crude extracts of *Terminalia*, so that in the extracts these compounds seem to be more antibacterial than separately (Table 3).

Part of the prospective antibacterial effects of the methanolic soxhlet root extract of *T. brownii* might be due to ellagic acid, which was present in high concentrations (10.82 % peak area) in the extract along with ellagic acid glycosides (Fig. 2, Table 4). We found that pure ellagic acid exerted less antibacterial activity (MIC 125 and 250 µg/ml against *S. aureus* and *P. aeruginosa*, respectively) than the crude extract (MIC 39 µg/ml) (Table 3). In contrast to our results on the antibacterial activity of ellagic acid, Machumi et al. (2013) found that ellagic acid derivatives in the stem bark of *T. brownii*, such as di-galloyl-rhamnopyranosyl ellagic acid and diellagic lactone gave very good growth inhibitory effects against *P. aeruginosa*, demonstrating as low IC50 values as 8.8 and 8.4 µg/ml, respectively. Thus, it is possible that the sugar part in glycosidic ellagic acid derivatives as well as galloyl-groups are important for their antibacterial activity. This warrants further studies in the antibacterial activities of ellagic acid glycosides and other ellagic acid derivatives isolated from the root part of *T. brownii*, both in combinations and separately. Moreover, ellagic acid derivatives have also been demonstrated to have indirect antibacterial effects and ellagic acid derivatives from a fruit extract of *Terminalia bellerica* were found to inhibit quorum sensing (QS) of *P. aeruginosa* (Sarabhai et al., 2013). In this same publication, Sarabhai et al. (2013) found that *pure* ellagic acid did not exert much biofilm inhibiting effect, but most of the inhibiting effects were due to a *combination* of ellagic acid derivatives in *T. bellerica* fruit extract. Ellagic acid enriched extracts from *Terminalia* species could be a good source of growth inhibitory compounds suppressing virulence factors in *P. aeruginosa* and other bacteria.

According to our literature survey the roots of *T. brownii* are reported to be used for treatment of allergy in African traditional medicine (Kareru et al., 2007; Kareru et al., 2008; Mosango, 2013), but not for bacterial infections. Our results together with those of Mbwambo et al. (2007) indicate now that in addition to the stem bark and wood also the roots could be made into decoctions for treatment of bacterial infections. Aqueous extracts of the roots of *T. brownii* have been reported to be toxic to

brine shrimp larvae (Mbwambo et al., 2007), however, and therefore care should be taken when using decoctions of the roots of this plant.

Stem bark and wood extracts

We have found that various stem bark extracts of *T. brownii* gave promising antibacterial activities (Table 2 and 3). Methanolic soxhlet and aqueous extracts expressed MIC values of 78.12 and 156.25 µg/ml, respectively, against *M. luteus* and were also effective against *S. aureus* giving MIC values of 156.25 – 312.50 µg/ml against this bacterium (Table 3). Moreover, also an ethyl acetate extract of the stem bark gave good effects against *P. aeruginosa* (IZ: 26 mm) (Table 2). Our results are in agreement with Abd alla et al. (2013), Machumi et al. (2013) and Mbwambo et al. (2007), who found that various extracts of the stem bark of *T. brownii* give good antibacterial activities. In accordance with our results, Mbwambo et al. (2007) found that especially aqueous extracts of the stem bark of *T. brownii* give promising antimicrobial effects. Therefore the chemical composition of aqueous extracts of *T. brownii* stem bark should be investigated in depth. To the best of our knowledge there exists only one investigation on antimicrobial compounds in *T. brownii* stem bark. Machumi et al. (2013) demonstrated antimicrobial ellagic acid derivatives and pentacyclic triterpenoids in ethyl acetate extracts of *T. brownii* stem bark. Decoctions of the stem bark of *T. brownii* are used for diarrhoea and wounds among a plethora of other uses (Machumi et al., 2013; Neuwinger, 2000) and these uses are now justified by our results. Moreover, aqueous and methanol extracts show little or no cytotoxicity (Mbwambo et al., 2007; Yamauchi et al., 2016).

We found that ethyl acetate and methanolic soxhlet extracts of the stem wood showed good growth inhibitory effects against *M. luteus* and *S. epidermidis* (Table 3). Our results are in agreement with Muddathir and Mitsunaga (2013) who reported on good antibacterial effects of a stem wood extract of *T. brownii* against *Propionibacterium acnes*. Mbwambo et al. (2007) found that especially aqueous extracts of the stem wood gave good antibacterial effects.

3.1.3. Anogeissus leiocarpus

We have found that extracts of roots, stem bark and stem wood of *A. leiocarpus* exert significant antibacterial effects, the roots giving the lowest MIC values (Table 2 and 3). Similarly to *T. laxiflora* and *T. brownii* we found that the antibacterial effects of *A. leiocarpus* extracts are dose-dependent. Our results are in accordance with previous investigations on promising antimicrobial potential of this plant.

Table 3

Minimum inhibitory concentration (MIC) of extracts and their fractions of *Terminalia laxiflora, T. brownii* and *Anogeissus leicarpus* stem wood, stem bark and roots selected from an initial screening using agar diffusion. MIC values were also estimated for punicalagin, ellagic acid and gallic acid which were found to be present in some of the extracts. MIC values obtained using microdilution* and agar disc diffusion methods. Results in µg/ml.

Plant extracts and antibiotics	***M. luteus***	***S. aureus***	***S. epidermidis***	***P. aeruginosa***
***T. laxiflora*(S.W.):**				
$CHCl_3$	2500	1250	1250	2500
EtOAc	**312.50***	625.00*	1250*	**156.25**
Aqueous fraction	1250*	625.00*	625.00*	**312.50**
MeSox	**78.12***	**156.25***	**78.12***	625.00****
***T. laxiflora* (S.B.):**				
EtOAc	**312.50***	**312.50***	625.00*	**39.06**
MeSox	**78.12**	**78.12**	**78.12**	**312.50***
***T. laxiflora* (R):**				
HH2O	**39.06**	**39.06**	**39.06**	**39.06**
MeSox	**39.06**	**39.06**	**39.06**	**39.06**
acetone	**39.06**	**39.06**	**39.06**	**39.06**
EtOAc	**39.06***	**39.06**	**39.06***	**39.06**
***T. brownii* (S.W.):**				
EtOAc	**156.25***	625.00*	1250*	**312.50***
MeSox	**312.50***	**312.50***	**156.25**	**312.50**
***T. brownii* (S.B.):**				
Aqueous fraction	**156.25**	**312.50***	1250*	1250*
MeSox	**78.12***	**156.25**	**156.25***	**156.25**
***T. brownii* (R):**				
HH2O	**39.06**	**78.12**	**39.06**	**39.06**
MeSox	**39.06***	**39.06**	**39.06**	**39.06**
acetone	**39.06***	**39.06**	**39.06**	**312.50**
EtOAc	**39.06***	**39.06**	**78.12***	**156.25**
***T. brownii* MeSox (R):**				
Sephadex LH-20 EtOH wash	NT	93.75*	NT	187.50*
***A. leiocarpus* (S.B.):**				
EtOAc	625.00*	625.00*	**312.50***	**39.06**
Aqueous fraction	**78.12**	**312.5**	2500*	**39.06**
MeSox	**39.06**	**39.06**	**312.50***	625.00*
***A. leiocarpus* (S.W.):**				
MeSox	**78.12***	**156.25**	625.00*	1250*
***A. leiocarpus* (R):**				
HH2O	**39.06**	2500	1250	**78.12**
MeSox	**39.06**	**39.06**	**39.06**	**39.06**
acetone	**39.06**	**78.12**	**39.06**	**312.50**
EtOAc	**39.06***	**78.12**	**39.06**	**312.50***

Table 3
Continued.

Plant extracts and antibiotics	*M. luteus*	*S. aureus*	*S. epidermidis*	*P. aeruginosa*
Pure compounds:				
Punicalagin	NT	125.00*	NT	500.00*
Ellagic acid	NT	125.00*	NT	250.00*
Gallic acid	NT	500.00*	NT	250.00*
Antibiotics:				
Ampicillin	0.976*	0.488*	0.976*	NT
Gentamycin	NT	NT	NT	62.50*
Tetracycline	0.488*	0.031*	0.976*	125.00*****
Penicillin	0.061*	1.950*	0.061*****	NT

MIC as mean of triplicates; S.W., stem wood; S.B., stem bark; R, roots. MeSox, methanolic soxhlet extract; HH2O, decoction; EtOAc, ethyl acetate extract; $CHCl_3$, Chloroform extract; NT, not tested. The most promising results indicated in bold.

Root extracts

In agreement with some earlier investigations (Mann et al., 2008b; Ogundiya et al., 2006; Taiwo et al., 1999), we found that a methanolic soxhlet root extract of *A. leiocarpus* gave highly promising antibacterial effects, expressing MIC values of 39.06 µg/ml against all investigated bacteria (Table 3) and large inhibition zones of 36 and 28 mm, respectively, against *M. luteus* and S. *epidermidis* (Table 2). We found that this methanolic root extract is rich in flavonoids such as ampelopsin, aromadendrin, methyltaxifolin and its isomer as well as taxifolin and the stilbenes pinosylvin and methylpinosylvin (Table 5, Fig. 3, 5 and 8). The mentioned flavonoids and stilbenes have not been characterized earlier in the roots of *A. leiocarpus*. Moreover, we found several ellagic acid derivatives including di-methyl ellagic acid and its glucoside and xyloside in this extract (see chapter 3.2.3. for more information on the chemistry of *A. leiocarpus* roots). The excellent antibacterial effects of the methanolic soxhlet root extract might be due to some of the mentioned compounds.

Some of the flavonoids and related compounds we have found to be present in the roots of *A. leiocarpus* have been shown to exert promising antibacterial effects. Ampelopsin A and F gave some antibacterial effects (MIC 100-200 µg/ml) against *Streptococcus mutans* (Yim et al., 2010). Taxifolin was found to inhibit the growth of *Streptococcus sobrinus*, a bacterium common in dental plaques (Kuspradini et al., 2009). It is thought that the stereochemistry at C2 and C3 positions govern the antimicrobial potency of the flavanol taxifolin as well as in general for flavanones and flavan-3-ols (Cushnie and Lamb, 2011; Xu et al., 2013). Also, the position of hydroxyl and methyl moieties in flavonoids have been thought to be important for their antibacterial activity and hydroxylation at C2 in A ring and C3 in C ring has been found to increase their antimicrobial activity (Alvarez et al., 2004; Boulos et al., 2013; Cushnie and Lamb, 2011; Otsuka et al., 2008). Flavonoids could be powerful new antibacterial tools since they have been found to act synergistically with antibiotics and to suppress

bacterial virulence. The most antimicrobial flavonoid to date is Panduratin A which has given a MIC of 0.06 – 2 µg/ml against *S. aureus* (Cushnie and Lamb, 2011). Antibacterial flavonoids might have many cellular targets, rather than just one specific site of action as was thought previously. Flavonoids bind to proteins and thus their antimicrobial effects might be related to inactivation of microbial adhesins, cell envelope transport proteins and enzymes. Lipophilic flavonoids may disrupt microbial membranes (Kumar and Pandey, 2013).

Of the stilbenes we have found in *A. leiocarpus* roots, pinosylvin has been demonstrated to possess potent bactericidal activity which was higher than that shown by resveratrol (Silva et al., 2015a). Pinosylvin is included in the constitutive defense of plants against microorganisms and has been found to be especially effective as an antifungal compound against wood-destroying fungi (Hovelstad et al., 2006). Stilbenes are powerful antibacterial compounds since they act on bacterial membranes to interrupt their functions such as permeability and efflux actions (Silva et al., 2015a).To the best of our knowledge the other stilbene compound we have found in *A. leiocarpus* roots, methylpinosylvin, has not been subjected to antimicrobial testing to date. The good antibacterial effects we have found for the methanol root extract of *A. leiocarpus* could be due partly to the mentioned stilbenes. The antibacterial effects of methylpinosylvin should be elucidated.

We found that also ethyl acetate and acetone extracts of the roots of *A. leiocarpus* gave significant growth inhibitory results against *M. luteus* and *S. epidermidis* (MIC 39.06) and some inhibitory effects against *P. aeruginosa* (312.50 µg/ml) (Table 2 and 3). Moreover, the decoctions of the roots demonstrated promising antibacterial activity against *P. aeruginosa, M. luteus* and *S. aureus* giving MIC values of 39.06 – 78.12 µg/ml (Table 3). These low MIC values correlate well with the agar diffusion results of the decoction, expressing large inhibition zones against *P. aeruginosa* and *M. luteus* (Table 2).

The traditional uses of the roots of *A. leiocarpus* as antibacterial infusions and decoctions for acute respiratory tract infections (Rajpoot and Singh, 2014), as antibacterial chewing sticks (Gbadamosi and Ogunsuyi, 2014) as well as for treatment of wounds (Mann et al., 2008b) are now justified by our results. Our results warrant further research into isolation of antimicrobial flavonoids and stilbenes from *A. leiocarpus* roots. *A. leiocarpus* roots have not been explored sufficiently for new antimicrobially active compounds.

Stem bark extracts

We found that methanolic soxhlet, aqueous and ethyl acetate extracts of the stem bark expressed promising antibacterial effects (Table 2 and 3). Nonpolar extracts, such as chloroform and petroleum ether extracts, expressed just slight antibacterial effects (Table 2). The best effects were obtained with

ethyl acetate and aqueous extracts giving low MIC value of 39.06 µg/ml against *P. aeruginosa* and methanolic soxhlet extracts against *S. aureus* and *M. luteus* (Table 3). These results are in agreement with the investigations of Mann et al.'s (2008b, 2009, 2010, 2014) and Mann (2012), who found that stem bark extracts of *A. leiocarpus* give broad-range antimicrobial effects against Gram-positive and Gram-negative bacteria.

We found that the highly antibacterial ethyl acetate extract of the stem bark of *A. leiocarpus* is enriched in ellagitannins. In addition it contains some condensed tannins such as epigallocatechin gallate as well as some gallotannins (Table 5, Fig. 4, See paragraph 3.2.4. for details on chemistry). Therefore the good antibacterial activity of this extract could be due to its polyphenols. Recently, polyphenols including epigallocatechin have been drawing a great interest for their antibacterial potential against Gram-negative MDR bacteria (Betts and Wareham, 2014; Gordon and Wareham, 2010). In the presence of metal ions, polyphenols have been thought to function as pro-oxidants leading to increased production of H_2O_2 and formation of a free hydroxyl radical promoting outer membrane disruption and lysis in *Escherichia coli* and *Klebsiella pneumoniae* (Arakawa et al., 2004). These findings warrant further studies on the mode of action of polyphenols in combinations with other plant derived antimicrobials as well as with conventional antibiotics against human pathogenic bacteria (Daglia, 2012).

The powdered stem bark of *A. leiocarpus* is used for wounds, sores, cysts and as cough medicine in African traditional medicine (Mann et al., 2008b) and these uses are in agreement with our results on promising antibacterial activities of stem bark extracts of this plant.

Stem wood extracts

We report, in agreement with Akande and Hayashi (1998), that extracts of *Anogeissus leiocarpus* stem wood give antibacterial effects. Methanolic soxhlet extracts and aqueous fractions were especially active against *M. luteus* and *S. aureus* giving MIC values of 78.12 and 156.25 µg/ml, respectively, and large inhibition zones from 21-26 mm against all the investigated bacterial strains (Table 2 and 3). This indicates that polar compounds, such as ellagitannins could be the antibacterially active compounds in these extracts. Interestingly, the chloroform soluble extracts of the stem wood gave significant antibacterial effects against *P. aeruginosa* (IZ: 28 mm, Table 2), but were just slightly active against the Gram-positive bacterial strains, indicating that some more nonpolar compounds in *A. leiocarpus* stem wood could be effective inhibitors of Gram-negative bacteria. A petroleum ether extract of the stem wood gave very good growth inhibitory activity against *S. epidermidis* (IZ: 30 mm) and indicates that stem wood preparations mixed with animal fat could be used topically as ointments for treatment of bacterial infections on the skin. Triterpenes and some triterpene glycosides have been

found in the stem bark of *A. leiocarpus* (Chaabi et al., 2008) and triterpenoidal fractions of stem bark have been found to give good antibacterial activity (Mann et al., 2009). Triterpenoids might be responsible for the antibacterial effects we have observed for the more nonpolar extracts of the stem wood of *A. leiocarpus*. Rajpoot and Singh (2014) found some antibacterial triterpenoids from the stem of *A. leiocarpus*.

Our results support the use of whole twigs of *A. leiocarpus* as chewing sticks (Dweek, 1996). Tannins in these sticks might be the active ingredients and they do not need to be digested but can exert their effects directly by binding to harmful bacteria in the mouth. Staphylococci have been found to cause tonsillitis in the mouth in immunocompromised individuals, and our results thus confirms that chewing sticks of *A. leiocarpus* could be used to treat this condition. Moreover, the high content of ellagic acid derivatives in *A. leiocarpus* also might contribute to inhibit pathogenic bacteria in the mouth since ellagic acid isolated from Chinese gall, *Gala chinensis*, has been found to effectively inhibit the growth of *Streptococcus* species (Loo et al., 2010).

3.2. HPLC-DAD and UHPLC/Q-TOF MS results

Extracts expressing promising antibacterial activity where subjected to phytochemical investigations, with special emphasis on characterization of their ellagitannin and flavonoid composition. Ethyl acetate extracts of the root of *T. laxiflora* and methanolic soxhlet extracts of the root of *T. brownii* as well as methanolic root and ethyl acetate stem bark extracts of *A. leiocarpus* were found to contain ellagitannins, ellagic acid derivatives, flavonoids, stilbenes and phenolic acids. Many of the identified compounds have not been presented before in *Terminalia laxiflora*, *T. brownii* and *A. leiocarpus*. In addition a number of unknown ellagitannins are presented. Negative ESI-MS was found to be favourable for detection of ellagitannins in the extracts and was used for flavonoids and stilbenes as well

3.2.1. Terminalia laxiflora root

Our phytochemical analysis of an ethyl acetate extract of the root of *T. laxiflora* resulted in the identification of eighteen ellagitannins and two ellagic acid glycosides (Table 4, Fig. 1). This result is in agreement with Ekong and Idemudia (1967) who reported on ellagic acid derivatives in the heartwood of *T. laxiflora* and with Muddathir et al. (2013) who found ellagitannins in methanol extracts of *T. laxiflora* stem wood. To the best of our knowledge the polyhydroxy-lactone diterpene laxiflorin is the only compound known to date in the roots of *T. laxiflora* (Ekong and Idemudia, 1967) and therefore we have focused on the chemistry of the roots of this species of *Terminalia*.

We report here for the first time on the occurrence of the ellagitannins corilagin **(3)** (t_R 12.52 min, $[M-H]^-$ ion at m/z 633.0750) and its more polar isomer **(2)** (t_R 8.56 min, $[M-H]^-$ ion at m/z 633.0743) as well as punicalagin **(4)** (t_R 15.25 min, $[M-H]^-$ ion at m/z 1083.1541) (Table 4, Fig. 1, 5). We found that flavogallonic acid dilactone and terchebulin were absent from the roots of *T. laxiflora*, even though these ellagitannins are reported to occur in the stem wood of this plant (Muddathir et al., 2013). Corilagin and its isomer as well as punicalagin are present in high concentrations in *T. laxiflora* roots at 6.7, 20.4 and 13.0 % peak area, respectively, at 220 nm (Table 4, Fig.1). Corilagin and punicalagin along with their derivatives have been found in various organs of other *Terminalia* species, such as in the roots of *Terminalia macroptera* (Pham, 2014; Silva et al., 2000; Silva et al., 2012) and the fruits of *Terminalia horrida*, *T. bellerica* and *T. chebula* (Miyasaki et al., 2013; Pfundstein et al., 2010). Corilagin is a fairly rare ellagitannin, and has originally been found from *Caesalpinia coriaria* (Leguminosae) (Pham, 2014). In addition to the mentioned ellagitannins we identified two unknown ellagitannins (**7** and **8**) in the roots of *T. laxiflora* at t_R 27.88 and 29.73 min, giving $[M-H]^-$ molecular ions of 817.4026 and 817.4033, respectively (Table 4, Fig. 1). For these ellagitannins we could not find references in literature, and therefore NMR analysis should be made to confirm their chemical identity. A number of other ellagitannins were also identified (Table 4, Fig. 1) according to their characteristic UVλ absorbance maxima showing three peaks of maximum absorbance, but the molecular masses were not detected for these.

Ellagic acid-xylopyranoside **(5)** and methyl ellagic acid xyloside **(6)** giving $[M-H]^-$ molecular ions of 433.0407 and 447.0568, respectively, have not been presented before in the roots of *T. laxiflora* (Table 4, Fig. 1, 5). Trimethyl- and tetramethyl ellagic acid have been reported earlier in the heartwood of *T. laxiflora* (Ekong and Idemudia, 1967). Mono- and dimethyl ellagic acid as well as gallagic acids which are building blocks of ellagitannins are known from many species of *Terminalia* (Machumi et al., 2013; Pfundstein et al., 2010; Silva et al., 2015b).

3.2.2. Terminalia brownii root

From quadrupole time of flight mass-spectrometric and HPLC-DAD analysis, eleven ellagitannins, two gallotannins, gallic acid and seven ellagic acid derivatives along with pure ellagic acid were characterized from a methanolic soxhlet extract of the root of *T. brownii* (Table 4, Fig. 2). To the best of our knowledge there is only one previous investigation on ellagitannins in the stem bark of *T. brownii* (Yamauchi et al., 2016), but there are no previous reports on the ellagitannin composition of the root. Therefore, our research has focused especially on ellagitannins in the root part of this plant, and the $[M-H]^-$ molecular ions for nine ellagitannins are presented in Table 4. We characterized the

ellagitannin methyl-(S)-flavogallonate **(3)** (t_R 12.63, $[M-H]^-$ molecular ion at m/z 483.0795) and its more polar isomer **(2)** (t_R 8.64, $[M-H]^-$ molecular ion at m/z 483.0811) (Fig. 2, 5) from a root extract of *T. brownii*. Methyl-(S)-flavogallonate has been identified previously in the fruits of *Terminalia chebula*, *T. bellerica* and *T. horrida* (Pfundstein et al., 2010) as well as in *Terminalia myriocarpa* leaves (Marzouk et al., 2002). The other ellagitannins present in the roots of *T. brownii* showed $[M-H]^-$ molecular ions ranging from m/z 456.9961-817.3999, two of the structures being isomers (negative ions at 577.1369 and 577.1320) showing the same retention time of 35.75 min (Table 4). Consequently, further studies using NMR are required to determine their chemical structures. It has been discovered that ellagitannins with the same exact mass may have several isomeric structures (Pfundstein et al., 2010).

We identified seven glycosidic derivatives of ellagic acid in the roots of *T. brownii*. Di-methyl-ellagic acid-di-xyloside **(4)** (tR 14.82, $[M-H]^-$ molecular ion at m/z 595.0950 and MS/MS fragment ion at m/z 463.0537 which corresponded to the loss of 132 from the molecular ion, the MW of xylose), ellagic acid glucuronide (**6a**) (tR 17.85, $[M-H]^-$ molecular ion at m/z 477.0651), methyl ellagic acid glucuronide **(6b)** (tR 17.85, $[M-H]^-$ molecular ion at m/z: 491.0829), ellagic acid xylopyranoside (**7**) (tR 18.27, $[M-H]^-$ molecular ion at m/z 433.0409) and methyl ellagic acid xyloside (**8**) (tR 19.39, $[M-H]^-$ molecular ion at m/z 447.0569) were identified for the first time from the roots of *T. brownii* (Table 4, Fig. 2, 5). The last two mentioned ellagic acid glycosides (**7** and **8**) occur also in the root of *T. laxiflora* (Table 4). Ellagic acid glucuronides have not to the best of our knowledge been previously identified in *Terminalia* spp.. Xylose based glycosides of mono- and dimethyl ellagic acids are known from the fruits of *Terminalia bellerica*, *T. horrida* and *T. chebula* (Pfundstein et al., 2010; Sarabhai et al., 2013) as well as from the stem bark of *Terminalia superba* (Kuete et al., 2010). Recently, ellagic acid and several ellagic acid derivatives, among them 3-*O*-methylellagic acid, 3,3′-di-*O*-methylellagic acid, ellagic acid 4-O-α-L-rhamnopyranoside and 3,3′,4-tri-*O*-methylellagic acid have been found in the stem bark of *Terminalia brownii* (Machumi et al., 2013; Yamauchi et al., 2016).

Along with gallic acid **(1)** (tR 1.74, MW: 170.0213) we identified a gallotannin in the roots of *T. brownii* (tR 38.98, $[M-H]^-$ molecular ion at m/z 553.1732) (Table 4, Fig. 2). To the best of our knowledge there are no previous reports on gallotannins in *T. brownii*.

3.2.3. Anogeissus leiocarpus root

To the best of our knowledge mass spectrometric analysis has not been conducted before on compounds present in the the root of *A. leiocarpus*. Therefore a methanolic soxhlet extract was analyzed for its phenolic compounds using HPLC-DAD and UHPLC/Q-TOF MS. A total of twenty-four compounds were identified (Table 5, Fig. 3). Among these compounds eight ellagic acid

derivatives, two phenolic acids (gallic and protocatechuic acid), two stilbenes and five flavonoids were detected, and some of them have not previously been found in the roots of *A. leiocarpus*. Previously reported phenolic acids in *A. leiocarpus* include chebulagic, chebulinic, gentisic and protocatechuic acid (Arbab, 2014).

We report here on the occurrence of the flavonols aromadendrin syn. dihydrokaempferol **(3)** ([M-H]$^-$ molecular ion at m/z 288.0000), ampelopsin **(5)** ([M-H]$^-$ molecular ion at m/z 319.1417), taxifolin **(7)** ([M-H]$^-$ molecular ion at m/z 303.0365), methyltaxifolin **(6a)** ([M-H]$^-$ molecular ion at m/z 317.0678) and its isomer **(6b)** ([M-H]$^-$ molecular ion at m/z 317.0676) in the roots of *A. leiocarpus* (Table 5, Fig. 3, 5 and 8). Previously, eight flavonoids such as 4H-1-Benzopyran-4-one, 7-[(6-deoxy-α-Lmannopyranosyl)oxy]-5-hydroxy-2-(4-hydroxy-3-methoxyphenyl), catechin, quercetin, isoquercetin, rutin, vitexin and kaempferol have been reported in *A. leiocarpus* leaves (Arbab, 2014).

In addition, we identified the stilbenes, pinosylvin syn. trans-3,5-dihydroxystilbene **(12)** ([M-H]$^-$ molecular ion at m/z 211.1339) and methylpinosylvin syn. 3,5-dimethyl-cis-stilbene **(15)** ([M-H]$^-$ molecular ion at m/z 225.0373) for the first time in the roots of *Anogeissus leiocarpus* (Table 5, Fig. 3, 5 and 8). Pinosylvin is a well known constituent in pine species such as heartwood of *Pinus sylvestris* and needles of *P. densiflora* (Lee et al., 2005) and its occurrence has been reported to be restricted to the genus *Pinus* (Dinelli et al., 2006). Stilbenes in general are reported to be common in the heartwood of the plant families Pinaceae, Myrtaceae and Moraceae. In Combretaceae (order Myrtales) several stilbenes, such as the combretastatins in *Combretum* (Pettit et al.'s 1987, 1988, 1989) and resveratrol and its glycosides in *Terminalia sericea* (Joseph et al., 2007) have been reported. Pterostilbene, a cytotoxic dimethylated derivative of resveratrol, was characterized from *Anogeissus acuminata* stem bark (Rimando et al., 1994) but has not been reported in *A. leiocarpus*.

Moreover, we identified four ellagic acid derivatives from the roots of *A. leiocarpus*, such as di-methyl ellagic acid glucoside **(8)** ([M-H]$^-$ molecular ion at m/z 491.0843), di-methylellagic acid xylopyranoside **(10)** ([M-H]$^-$ molecular ion at m/z 461.0739) and its aglycone **(11)** ([M-H]$^-$ molecular ion at m/z 329.0318) and an acetylated ellagic acid derivative **(13)** ([M-H]$^-$ molecular ion at m/z 343.0477) (Table 5).

3,3´-Di-O-methyl ellagic acid glucoside has been identified in *Terminalia paniculata* (Row and Rao, 1962) and 3,3´-Di-O-methyl-ellagic acid xyloside in *T. horrida*, *T. arjuna* and *T. bellerica* fruits (Pfundstein et al., 2010) as well as in the stem bark of *Anogeissus leiocarpus* (Hubert et al., 2014).

In addition to the above mentioned compounds we identified the gallotannin di-galloyl-β-D-glucose **(4)** ([M-H]$^-$ molecular ion at m/z 483.0808, MW 484.0846) in the roots of *A. leiocarpus* (Table 5). This gallotannin has not been identified earlier in *A. leiocarpus.*

Table 4

HPLC-DAD and UHPLC/Q-TOF MS data of ellagitannins, ellagic acid derivatives and gallotannins in the roots of *Terminalia brownii* and *T. laxiflora*.

***Terminalia laxiflora*; EtOAc extracts of the roots**	**Molecular formula**	**Rt HPLC-DAD (min)**	**Rt UHPLC (min)**	**$[M-H]^-$**	**Exact mass (calc.)**	**UVλ absorbtion max. from HPLC-DAD**	**Peak area (%)**
Monogalloylglucose	$C_{13}H_{16}O_{10}$	1,01		331,0000	332,0738	216, 276	0,4322
Gallic acid	$C_7H_6O_5$	1,71	1,27	169,0141	170,0213	216, 272	2,1671
ellagitannin		3,08				216, 260, 378	0,9178
ellagitannin		7,57				214, 260, 380	0,9898
ellagitannin		8,00				216, 258, 380	4,5748
Corilagin derivative		8,56	3,75	633,0743		216, 256, 374	20,4186
ellagitannin		9,12				216, 258, 374	2,4147
ellagitannin		9,89				216, 260, 380	0,8927
ellagitannin		10,64				210, 254, 382	0,5800
Trigalloylglucose	$C_{27}H_{24}O_{18}$	10,95				216, 275	4,6583
ellagitannin		11,15				216, 261	2,2674
ellagitannin		11,45				216, 258, 377	0,6582
ellagitannin		11,73				220, 256, 380	1,2851
ellagitannin		12,22				216, 256, 380	1,4657
Corilagin	$C_{27}H_{22}O_{18}$	12,52	4,42	633,0750	634,0798	216, 256, 380	6,6912
ellagitannin with gallic and ellagic acid units		13,28				218, 275	0,2469
ellagitannin with gallic and ellagic acid units		13,46				218, 263, 380	0,6119
Punicalagin	$C_{48}H_{28}O_{30}$	15,25	5,42	1083,1541	1084,0654	216, 256, 380	12,9913
Gallotannin		16,38				218, 262, 376	0,3532
ellagitannin		17,52				256, 380	0,3108
ellagic acid xylopyranoside	$C_{19}H_{14}O_{12}$	18,03	7,88	433,0407	434,0480	210, 255, 380	2,3893
methyl ellagic acid xyloside	$C_{20}H_{16}O_{12}$	20,48	8,23	447,0568	448,0636	254, 368	8,8803
ellagitannin		27,88	12,79	817,4026		220, 264, 398	0,9001
ellagitannin		29,73	13,50	817,4033		220, 254, 362	0,6765

Table 4
Continued.

***Terminalia brownii*; MeOH Soxhlet extract of the roots**	**Molecular formula**	**Rt HPLC-DAD (min)**	**Rt UHPLC (min)**	**$[M-H]^-$**	**Exact mass (calc.)**	**UVλ absorbtion max. from HPLC-DAD**	**Peak area (%)**
gallic acid	$C_7H_6O_5$	1,74	1,11	169,0136	170,0213	216, 272	1,2052
ellagitannin		3,75				216, 258, 381	1,2368
ellagitannin		5,80	6,28	456,9961		216, 256, 374	6,7846
ellagitannin		8,05				216, 258, 382	1,4888
Isomer of Methyl-(S)-flavogallonate		8,64	8,52	483,0811		216, 258, 375	7,6436
gallotannin		9,86				216, 272, 380	1,5824
Methyl-(S)-flavogallonate	$C_{22}H_{12}O_{13}$	12,63	8,60	483,0795	484,0273	216, 257, 381	2,2440
Di-methyl-ellagic acid-di-xyloside		14,82	10,39	595,0950		254, 362	1,3049
ellagitannin		15,36	10,50	609,1088		216, 256, 381	12,8987
Ellagic acid glucuronide		17,85	11,63	477,0651		254, 368	0,6982
Methyl ellagic acid glucuronide		17,85	11,89	491,0829		254, 368	
Ellagic acid-xylopyranoside	$C_{19}H_{14}O_{12}$	18,27	12,10	433,0409	434,0480	256, 380	3,2320
Methyl ellagic acid xyloside	$C_{20}H_{16}O_{12}$	19,39	12,42	447,0569	448,0636	254, 362	1,5295
Ellagic acid	$C_{14}H_6O_8$	20,36	12,57	300,9983	302,0060	254, 366	10,8179
ellagic acid derivative		22,33	13,19	343,0466		248, 368	1,6932
ellagitannin		28,31	14,67	725,4141		210, 262, 370	1,1540
ellagitannin		28,93	14,98	817,4003		219, 254, 363	0,8172
acetylated ellagic acid derivative		33,94	16,10	343,0498		222, 348, 375	1,4695
ellagitannin		35,75	16,43	577,1369		214, 254, 372	1,7649
ellagitannin		35,75	16,65	577,1320		214, 254, 372	
ellagitannin		37,95	16,90	817,3999		216, 250, 372	0,2569
gallotannin		38,98	17,22	553,1732		218, 278	0,8886

Rt (min), retention times obtained from HPLC-DAD and UHPLC-DAD; $[M-H]^-$, base ion at negative mode. The % peak area was obtained from HPLC-DAD chromatograms at 220 nm.

3.2.4. Anogeissus leiocarpus stem bark

Our chemical profiling of the ethyl acetate extract of the stem bark of *A. leiocarpus* revealed the presence of seventeen compounds including gallic acid, the flavonol ampelopsin, six ellagic acid derivatives and six unknown ellagitannins (Fig. 4).

Three unknown ellagitannins were identified at m/z 453.1062, 833.4009 and 725.4177 as shown in Table 5. The ellagitannins castalagin (MW 934), flavogallonic acid, punicalagin and terchebulin have been identified in butanol and aqueous extracts of the stem bark of *A. leiocarpus*, but castalagin was found to be absent from an ethyl acetate extract (Shuaibu et al., 2008). This explains why we did not detect castalagin in the ethyl acetate extract of *A. leiocarpus* stem bark.

We report for the first time on the occurrence of the condensed tannin epigallocatechin gallate (at m/z 457.0768) in the stem bark of *A. leiocarpus* (Table 5). Previously, (-)-epigallocatechin and (+)-gallocatechin have been characterized in the stem bark of *A. leiocarpus* (Hubert et al., 2014).

Moreover, we identified two ellagic acid derivatives in the stem bark as di-methylellagic acid xylopyranoside and di-methyl ellagic acid. These compounds were also present in the roots of *A. leiocarpus* (Fig. 3) and have been mentioned before to occur in *A.leiocarpus* stem bark (Adigun et al., 2000; Chaabi et al., 2008). Other ellagic acid derivatives which have been reported in the stem bark of *A. leiocarpus* include 3,3´,4´tri-O-methylflavellagic acid, 3,4,3´-tri-O-methylflavellagic acid 4´-O-glucopyranoside and 3,3´,3´tri-O-methylflavellagic acid-4β-D-glucoside (Adigun et al., 200; Chaabi et al., 2008; Hubert et al., 2014).

4. Conclusions

In our search for antibacterially active extracts of the African medicinal plants *Terminalia laxiflora, Terminalia brownii* and *Anogeissus leiocarpus* we can confirm that polar methanolic soxhlet and ethyl acetate extracts as well as decoctions of the roots and stem bark give promising growth inhibitory effects against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The lowest recorded MIC values of 39.06 µg/ml are in accordance with other reports on promising antimicrobial potential of African species of *Terminalia* and *Anogeissus* (Conrad et al., 2001).

We found that antibacterial root and stem bark extracts of *T. brownii*, *T. laxiflora* and *A. leiocarpus* are rich in ellagitannins, ellagic acid derivatives, flavonoids and stilbenes. The ellagitannins corilagin and its isomer as well as punicalagin have not been reported previously in the roots of *T. laxiflora*. Likewise, the ellagitannin methyl-(S)-flavogallonate and its isomer are reported for the first time in the roots of *T. brownii*. These ellagitannins, along with several unknown ellagitannins, occur in high concentrations in the studied *Terminalia* species and might be the

principal antibacterial compounds in polar extracts of these species, such as traditionally made decoctions. The probability of finding new ellagitannin structures in species of *Terminalia* and *Anogeissus* is high, since ellagitannins are diverse to their chemical structure.

Antibacterial effects of *Terminalia* spp. and *Anogeissus* spp. extracts might be due to a combination of ellagitannins and other compounds in the extracts (Miyasaki et al., 2013) and when these compounds are separated from each other their antimicrobial effects might decrease. Our study demonstrates that the pure compounds punicalagin, ellagic acid and gallic acid, which are present in the extracts of the studied plants, are less antimicrobial than the crude extracts. Moreover, a Sephadex LH-20 fraction of *T. brownii* roots, enriched with methyl-(S)-flavogallonate and its isomer, was found to give a higher MIC (93–187 µg/ml) compared to the crude extract (39 µg/ml) and the pure compounds (125–500 µg/ml). This implies that the compounds in the crude root extract of *T. brownii* act synergistically with each other.

Our results justify the traditional uses of hot water decoctions of *Terminalia laxiflora*, *T. brownii* and *Anogeissus leiocarpus* roots and stem bark in Africa for treatment of bacterial infections related to wounds and diarrhoea. Especially in remote regions in African countries lacking Western clinics and where conventional antibiotics can be difficult to find or too expensive to purchase there would be a need for new antimicrobial standardized extracts containing a known composition of active compounds (Eloff and McGaw, 2006). Further work should be performed in detail on the phytochemistry of various species of *Terminalia* and *Anogeissus* used in traditional medicine.

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Table 5

HPLC-DAD and UHPLC/ Q-TOF MS data of flavonoids, stilbenes, ellagitannins and ellagic acid derivatives in the roots and stem bark of *A. leiocarpus*.

***Anogeissus leiocarpus*; MeOH Soxhlet extract of the roots**	**Molecular formula**	**Rt HPLC-DAD (min)**	**Rt UHPLC (min)**	**$[M-H]^-$**	**Exact mass (calc.)**	**UVλ absorbtion max. from HPLC-DAD**	**Peak area (%)**
Gallic acid	$C_7H_6O_5$	1,71	1,24	169,0145	170,0213	216, 272	3,2040
Protocatechuic acid	$C_7H_6O_4$	3,91	1,34	153,0196	154,0264	218, 220, 260, 294	1,1163
ellagitannin		8,99				214, 256, 376	1,3816
Dihydrokaempferol (syn. Aromadendrin)	$C_{15}H_{12}O_6$	10,47	3,80	288,0000	288,0630	210, 288	0,9248
Di-galloyl-β-D-glucose	$C_{20}H_{20}O_{14}$	11,43	4,51	483,0808	484,0846	216, 276	1,7803
Ampelopsin (syn. Dihydromyricetin)	$C_{15}H_{12}O_8$	12,66	4,84	319,1417	320,0528	210, 292	5,4055
Methyltaxifolin (syn. (+)-Dihydroisorhamnetin)	$C_{16}H_{14}O_7$	13,44	5,50	317,0678	318,0735	210,294	2,3772
Isomer of Methyltaxifolin	$C_{16}H_{14}O_7$	14,82	5,98	317,0676	318,0735	210, 230, 288	2,6073
Taxifolin (syn. Dihydroquercetin)	$C_{15}H_{12}O_7$	17,05	6,45	303,0365	304,0579	210, 230, 290	5,9525
ellagic acid derivative		18,65				254, 380	1,7024
ellagic acid derivative		19,20				248, 370	1,7194
ellagic acid derivative		20,06				254, 362	1,5205
Di-methyl ellagic acid glucoside	$C_{22}H_{20}O_{13}$	21,17	7,75	491,0843	492,0897	254, 368	2,1967
Pentagalloylglucose	$C_{41}H_{32}O_{26}$	22,24		939,6700	940,1170	218, 284	0,6405
Di-methyl ellagic acid-xylopyranoside	$C_{21}H_{18}O_{12}$	24,67	9,65	461,0739	462,0792	246, 368	11,1780
ellagic acid derivative		25,08				250, 372	
ellagitannin		26,10				222, 250, 369	0,7353
Di-methyl ellagic acid	$C_{16}H_{10}O_8$	29,45	11,25	329,0318	330,0372	248, 376	10,2240
ellagitannin		31,24				250, 378	0,2985
ellagitannin		31,58				248, 374	
Pinosylvin syn.trans-3,5-Dihydroxystilbene	$C_{14}H_{12}O_2$	32,92	12,05	211,1339	212,0834	218, 308, 320	0,8785
Acetylated ellagic acid derivative		34,29	12,80	343,0477		222, 248, 370	1,8107
Ellagic acid derivative		36,39	13,43	359,0430		246, 380	5,2358
4`-Methylpinosylvin syn. 3,5 dimethyl-cis-stilbene	$C_{15}H_{14}O_2$	45,65	17,94	225,0373	226,0990	216, 218, 308, 318	19,0662

Table 5
Continued.

***Anogeissus leiocarpus*; EtOAc extract of the stem bark**	**Molecular formula**	**Rt HPLC-DAD (min)**	**Rt UHPLC (min)**	**$[M-H]^-$**	**Exact mass (calc.)**	**UVλ absorbtion max. from HPLC-DAD**	**Peak area (%)**
Gallic acid	$C_7H_6O_5$	1,68	0,95	169,0139	170,0213	216, 272	5,6432
ellagitannin		8,48	8,32	453,1062		217, 256, 374	3,3271
Ampelopsin	$C_{15}H_{12}O_8$	11,70	4,72	319,1417	320,0528	210, 292	0,6719
Epigallocatechin gallate	$C_{22}H_{18}O_{11}$	13,70	9,94	457,0768	458,0843	210, 275	4,2513
ellagitannin		15,03				216, 256, 375	0,9794
ellagitannin		15,50				210, 256, 366	1,4000
Gallotannin		16,70	11,34	441,0852		216, 270	1,3627
ellagic acid derivative		18,02				255, 380	2,0827
ellagitannin		19,47				210, 254, 362	0,4205
Di-methyl-ellagic acid xylopyranoside	$C_{21}H_{18}O_{12}$	20,49	13,06	461,0766	462,0792	254, 369	3,0833
ellagic acid derivative		23,91	13,92	673,1030		246, 370	2,1831
ellagitannin		26,92	14,47	833,4009		210, 248, 370	1,3134
Di-methyl ellagic acid	$C_{16}H_{10}O_8$	28,78	14,89	329,0000	330,0372	248, 376	6,2357
ellagic acid derivative		30,92	15,56	613,0858		248, 378	0,5120
ellagitannin		33,85	16,49	725,4177		219, 248, 370	2,4310
ellagic acid derivative		35,76	16,72	359,0436		246, 381	2,9099

Rt (min), retention times obtained from HPLC-DAD and UHPLC; $[M-H]^-$, base ion at negative mode. The % peak area was obtained from HPLC-DAD chromatograms at 220 nm.

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Figure captures for this research paper:

Fig. 1. HPLC fingerprint at 220 nm of an antibacterial (MIC 39.06 µg/ml against all tested bacteria) ethyl acetate extract of the roots of *Terminalia laxiflora*. Gallic acid **(1)** and the ellagitannins corilagin **(3)** and its isomer **(2)** as well as punicalagin **(4)** occur in high concentrations in this extract. Ellagic acid -xylopyranoside **(5)** and methyl ellagic acid xyloside **(6)** are reported for the first time in the root of *T. laxiflora*. Two large-molecular, nonpolar ellagitannins **(7** and **8)** were present at 27.886 and 29.733 min. UVλ absorbtion maxima are indicated for compounds **2**, **3**, **4** and **5**.

Fig. 2. HPLC fingerprint at 270 nm of an antibacterial (MIC 39.06 µg/ml) soxhlet methanol extract of the roots of *Terminalia brownii*. Gallic acid **(1)** and eleven ellagitannins were identified in this extract, among them the possible isomer of methyl-(S)-flavogallonate **(2)**, methyl-S-flavogallonate **(3)** and an unknown ellaggitannin **(5)**. In addition this extract contains di-methyl-ellagic acid-di-xyloside **(4)**, ellagic acid glucuronide **(6a)** and its methylated derivative **(6b)**, ellagic acid xylopyranoside **(7)**, methyl ellagic acid xyloside **(8)**, ellagic acid **(9)**, an ellagic acid derivative **(10)** and an acetylated ellagic acid derivative **(13)**. **11**-**12** and **14**-**15** are ellagitannins. **16** is a gallotannin. UVλ absorbtion maxima are indicated for compounds **3**, **4**, **7** and **8**.

Fig. 3. HPLC fingerprint at 270 nm of an antibacterial methanolic soxhlet extract of the roots of *Anogeissus leiocarpus*. This extract contains the flavonoids aromadendrin **(3)**, ampelopsin **(5)**, methyltaxifolin **(6a)** and its isomer **(6b)** and taxifolin **(7)**. In addition this extract contains the phenolic acids gallic acid **(1)** and protocatechuic acid **(2)**. Among gallotannins, Di-galloyl-β-D-glucose **(4)** and penta-galloyl-glucose **(9)** were detected. Several ellagic acid deriavtives, including di-methyl ellagic acid glucoside **(8)**, di- methylellagic acid -xylopyranoside **(10)**, di-methyl-ellagic acid **(11)**, an acetylated ellagic acid derivative **(13)** and an ellagic acid derivative **(14)** were identified. Two stilbenes, pinosylvin **(12)** and methylpinosylvin **(15)** were identified for the first time from the roots of *A. leiocarpus*. UVλ absorbtion maxima are indicated for compounds **5**, **7**, **12** and **15**.

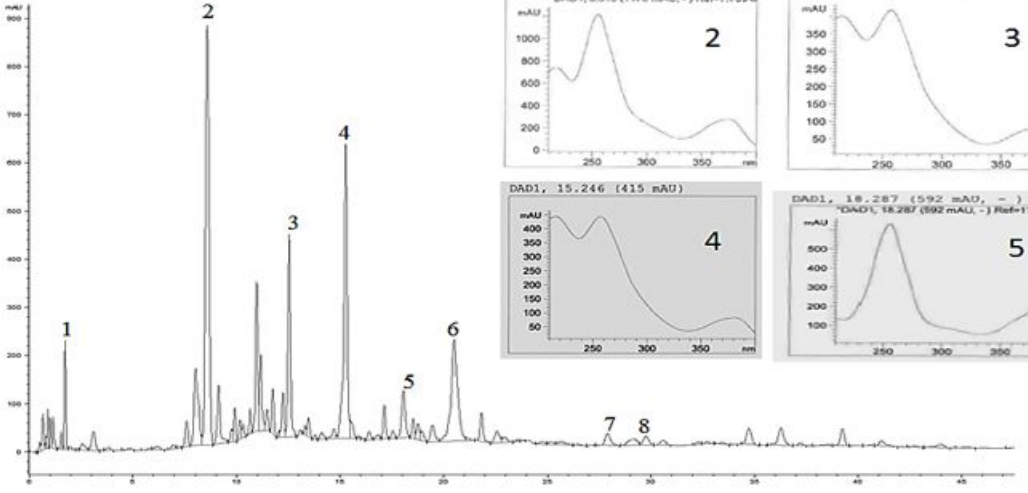
Fig. 4. HPLC fingerprint at 270 nm of an antibacterial ethylacetate extract of the stem bark of *Anogeissus leiocarpus*. This extract contains the gallic acid (**1**), ampelopsin (**3**), epigallocatechin gallate (**4**), di- methyl ellagic acid xylopyranoside (**9**), ellagic acid derivatives (**8a**, **13, 15**) and di- methyl ellagic acid (**12**) along with six unknown ellagitannins (**2, 5, 6**, **8b**, **11** and **14**) and a gallotannin (**7**). UVλ absorbtion maxima are indicated for compounds **2**, **9**, **12** and **14**.

Fig. 5. Ellagitannins, ellagic acid derivatives, flavonoids and stilbenes found in this investigation in roots of *T. laxiflora**, *T. brownii* ** and stem bark and roots of *A. leiocarpus****.

Fig. 6. Concentration dependent inhibition of *T. laxiflora* root decoction against *M. luteus*. Results obtained with an agar diffusion method.

Fig. 7. Concentration dependent inhibition of a wood ethyl acetate extract of *T.laxiflora* against *S. aureus*. Results obtained with a microplate method.

Fig. 8. Mass spectra of a) pinosylvin and b) taxifolin present in the roots of *Anogeissus leiocarpus*.


1
2
3
4
5
6
7
8
DAD1, 8.619 (1176 mAU, -) Ref
*DAD1, 8.619 (1176 mAU, -) Ref=7.759
DAD1, 12.506 (391 mAU, -) Ref
*DAD1, 12.506 (391 mAU, -) Ref=11.939
DAD1, 15.246 (415 mAU)
DAD1, 18.287 (592 mAU, -) Ref
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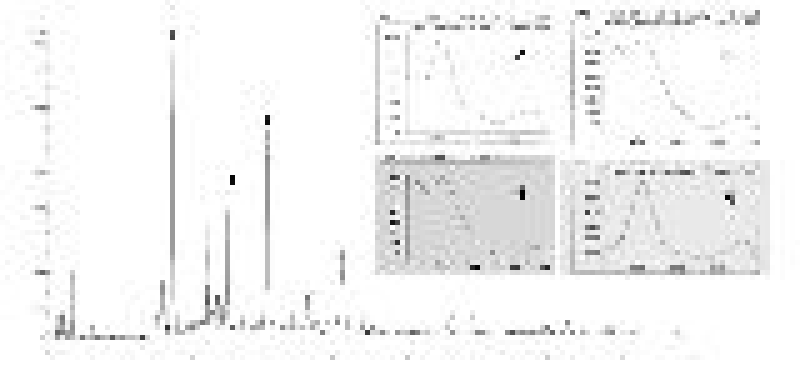
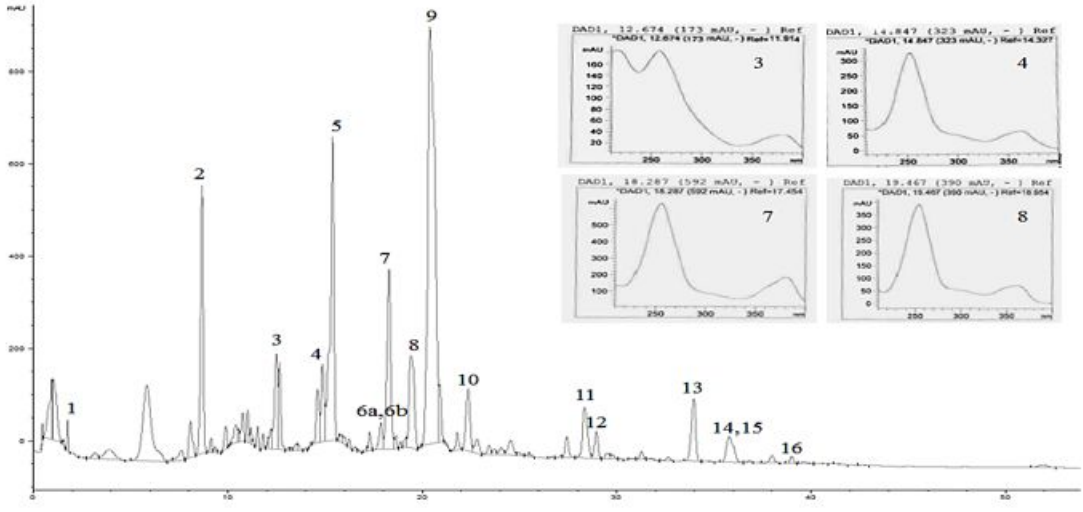
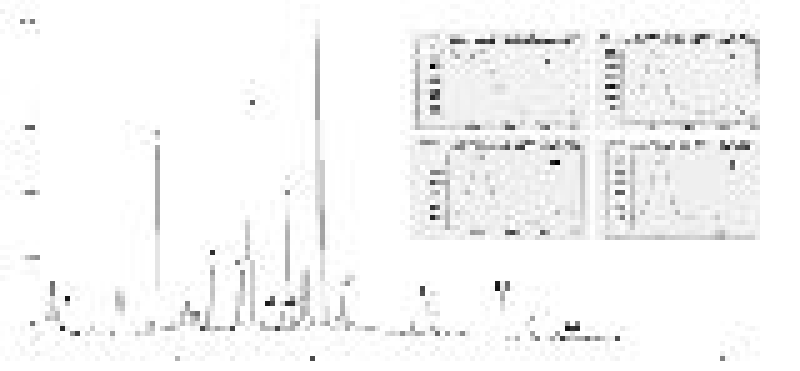
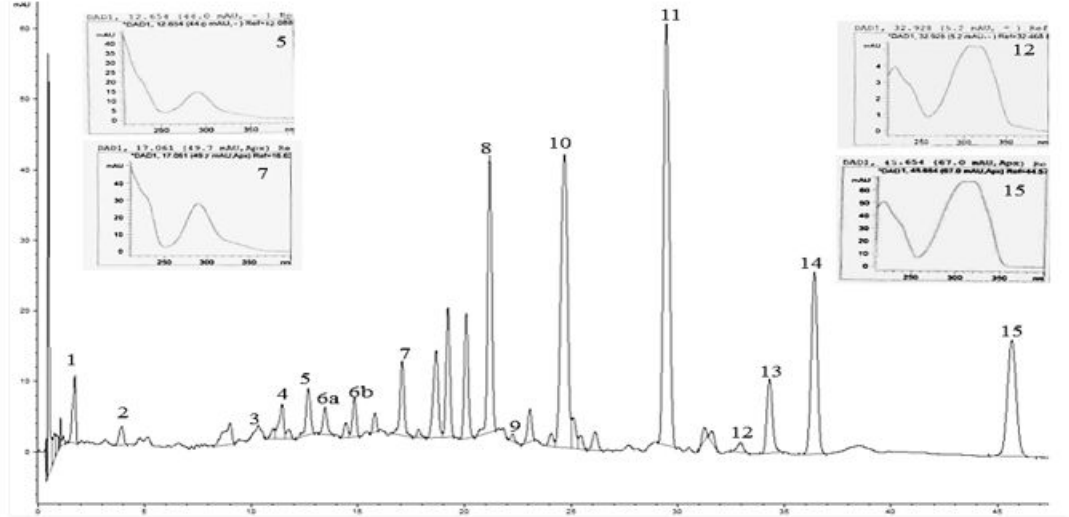



mAU
1
2
3
4
5
6a,6b
7
8
9
10
11
12
13
14,15
16
3
4
7
8




1
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6a
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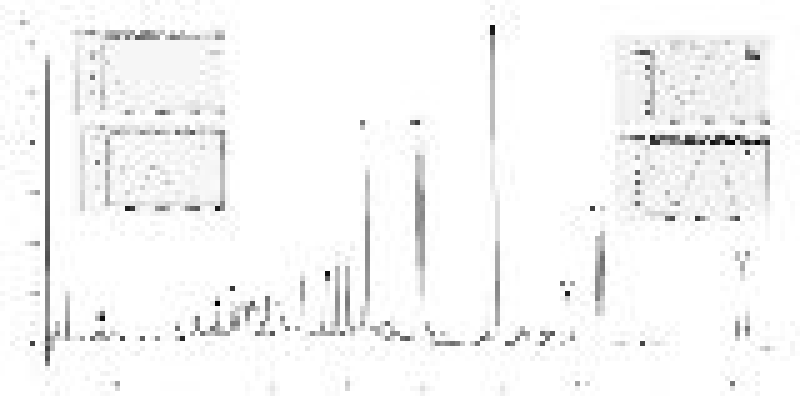

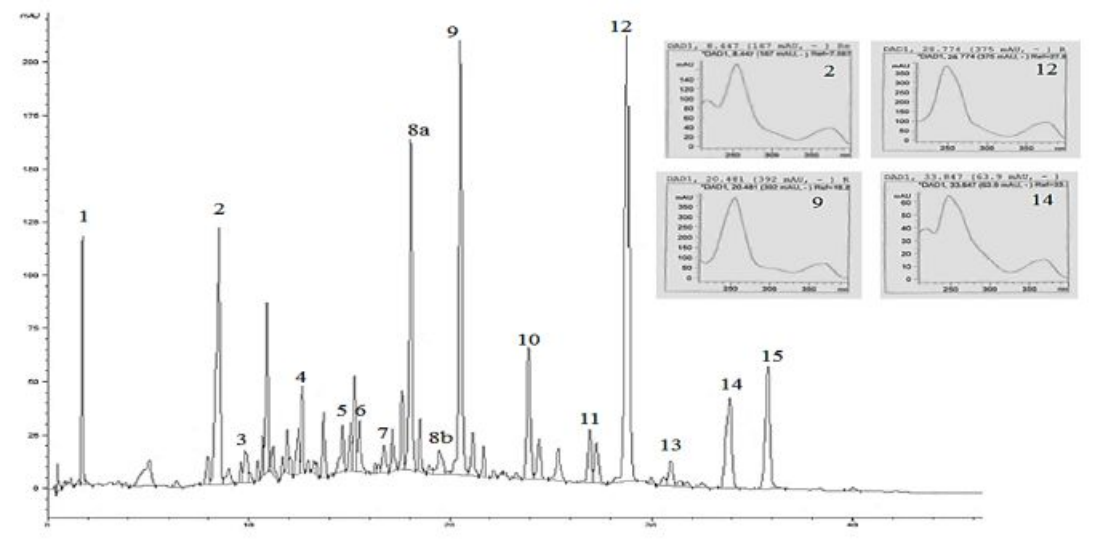
1
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8a
8b
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10
11
12
13
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15
2
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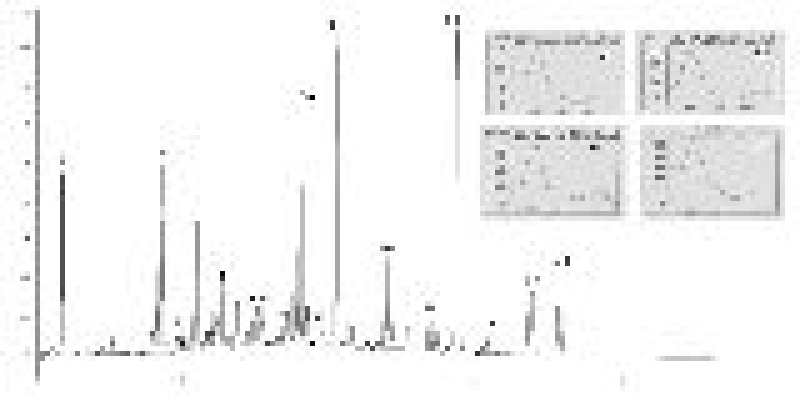
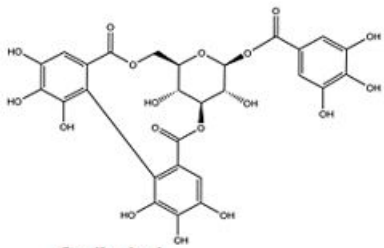
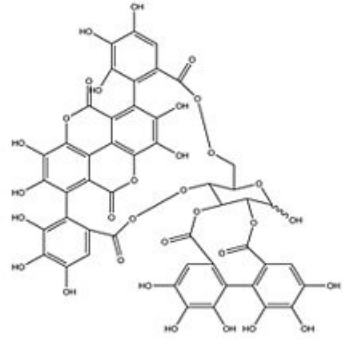
9
14

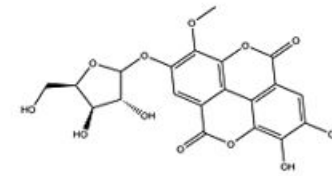




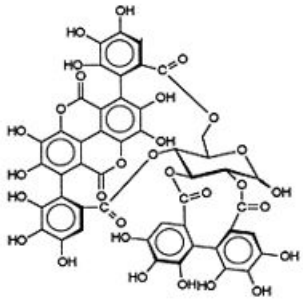
Corilagin *



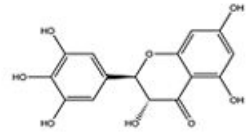
Punicalagin *



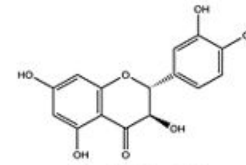
3- Methyl ellagic acid xyloside *, **



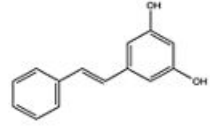
Methyl (S)-flavogallonate **



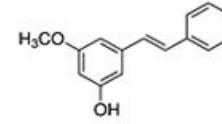
Ampelopsin ***



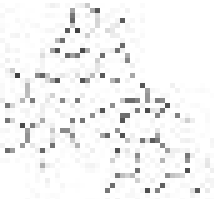
Taxifolin ***

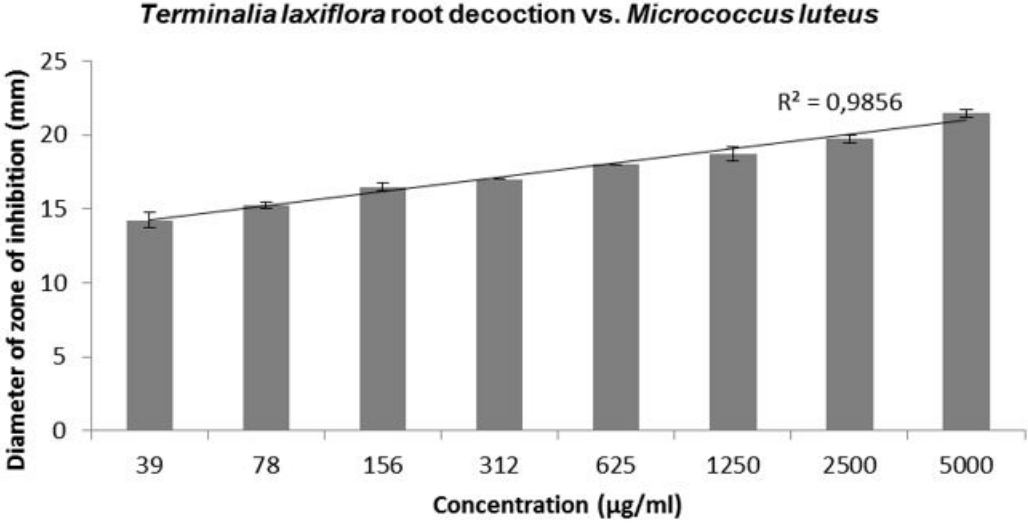


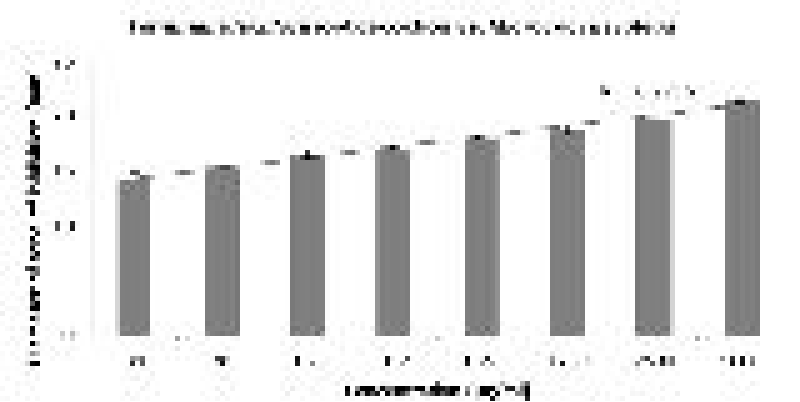
Pinosylvin ***

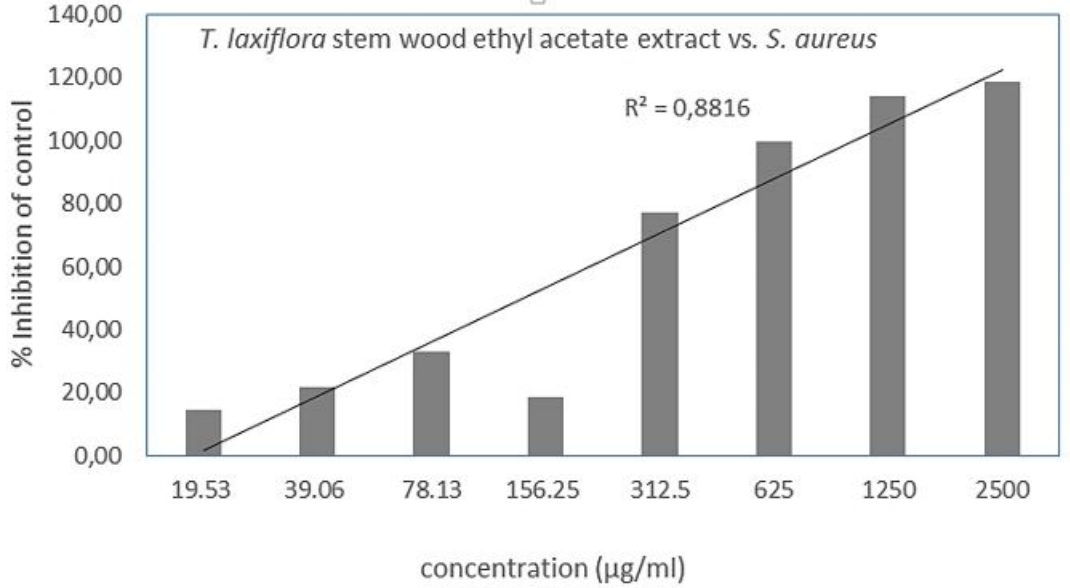


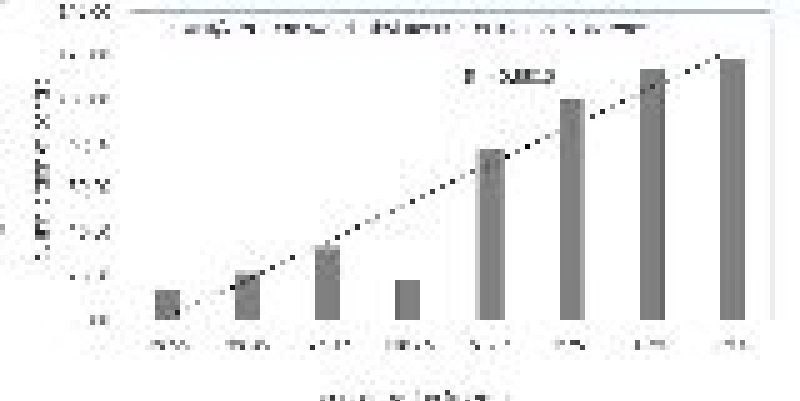
Methylpinosylvin ***



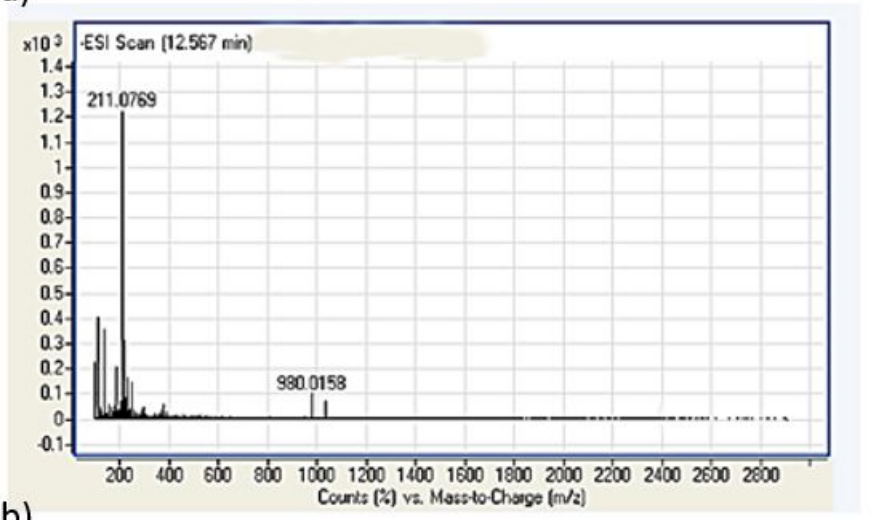

Terminalia laxiflora root decoction vs. Micrococcus luteus
R² = 0,9856
Diameter of zone of inhibition (mm)
25
20
15
10
5
0
39
78
156
312
625
1250
2500
5000
Concentration (µg/ml)




T. laxiflora stem wood ethyl acetate extract vs. S. aureus
R² = 0,8816
% Inhibition of control
140,00
120,00
100,00
80,00
60,00
40,00
20,00
0,00
19.53
39.06
78.13
156.25
312.5
625
1250
2500
concentration (µg/ml)



a)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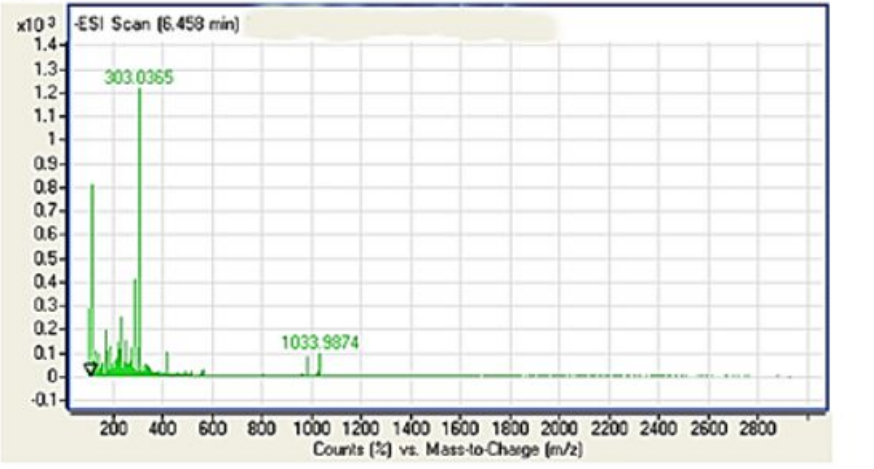
211.0769
980.0158
Counts (%) vs. Mass-to-Charge (m/z)

b)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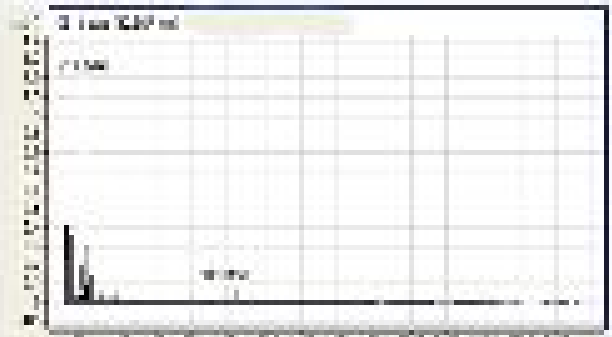
-ESI Scan (6.458 min)
303.0365
1033.9874
Counts (%) vs. Mass-to-Charge (m/z)

a.



b.