Chemical analysis and Biological properties of *Terminalia macroptera* Guill. & Perr. from Eastern Chad

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Abstract: Based on a previous ethnobotanical survey, *Terminalia macroptera* Guill. & Perr. was selected as its heartwood is traditionally used for infections treatment and as mosquito repellent in the province of Ouaddai (Chad). In our course for a better knowledge of this medicinal plant which could be developed as phytomedicine, it is of great interest to subsequently characterize the chemical profile and bioactivities of this plant. In the present study we develop an Ultrahigh-performance liquid chromatography with diode array detection coupled to electrospray ionization and quadrupole time-of-flight (UHPLC-DAD-ESI/QTOF) technique to characterized on line potentially bioactive compounds. Thirteen compounds were identified in active extracts and fractions from *Terminalia macroptera* Guill. & Perr. heartwood, namely gallic and ellagic acids derivatives, 23-O-galloyl-terminolic acid, 23-O-galloyl arjunolic acid, 4,4’-dihydroxy-Z-stilbene-O-rutinoside and 3,5,4’-trihydroxy-Z-stilbene-O-rutinoside. Antibacterial properties were evaluated against sensitive and resistant *Staphylococcus aureus* strains and antioxidant activities using 1,1-diphenyl-2-picryl-hydrayzyl (DPPH). Stilbene derivatives, 23-O-galloyl-terminolic acid and 23-O-galloyl arjunolic acid are newly reported in *T. macroptera* Guill. & Perr.

Keywords: Medicinal plant; *Terminalia macroptera* Guill. & Perr.; phytochemical analysis; UHPLC-DAD-ESI/QTOF; biological activities.

1. Introduction

Plants have long been used in all countries as natural medicines. In Africa, these natural medicines are a necessity due to the high cost of modern medicines. Eighty percent of African populations use traditional pharmacopoeia. Also, ensuring the quality and safety of these remedies, as well as the development of analytical methods to achieve this, becomes a requirement not to be circumvented. One of the most well-known traditional uses of plants is that of anti-infectious. The emergence of resistant bacteria to numerous antibiotics and the failure of conventional therapies have increased interest in the search for molecules or plant preparations that can counter these infections 1.

Based on an ethnobotanical survey, this study focused on *Terminalia macroptera* Guill. & Perr. (Fig.1) as a potential traditional medicine which chemical profiles need to be qualified 2. In the traditional medicine of Ouaddai (East of Chad), herbal tea of leaves of *T. macroptera* is drunk to treat hepatitis and the powder of dried roots is used for wounds healing 2. Heartwood of this plant is fumigated for infections healing and to keep mosquitoes away from homes. *T. macroptera* is used in other African countries to treat respiratory tract diseases, skin diseases, and wounds, hepatitis, malaria 3,4. This plant is used in West Africa for healing hepatitis, cough, tuberculosis, diarrhea, dysentery, fever, and malaria 3,4.

The genus *Terminalia* (Combretaceae) encounter around 200 different species growing in tropical area and in which at least 50 are used as food worldwide or as food supplements 7,8. Healers traditionally use others to treat coughs, bronchitis, dysentery, fractures, sores, ulcers, hypertension, and ischaemic heart diseases i.e. *T. arjuna* Wight & Arn., *T. catappa* Linn., *T. chebula* Retz. and *T. elliptica* Willd. 9,12. Many studies on these plants deal with the biological activities of extracts and pure compounds.

Antimicrobial, antimalaria, antioxidant, anticancer activities have been pointed out for extracts from leaves, barks, and roots of many of these species 13.
Some species, such as *T. chebula* or *T. arjuna* have been very well described.

However, fewer studies have been published on *T. macroptera* Guill. & Perr. [14-18]. Antioxidant activities of its root and stem barks extracts are reported using DPPH and ABTS reagents [19]. These radical scavenging activities are closely related to a high content in flavonoids and polyphenolic compounds. *T. macroptera* extracts are also reported to have interesting antiprotozoal properties against *Trypanosoma brucei brucei* [20]. Several studies recently enhanced the data on antimalaria activities of root and stemmed barks and leaves of *T. macroptera* [21-23].

Antimicrobial activities are the most investigated in Gram-positive and Gram-negative strains and on sensitive and resistant strains. Evaluation of the antistaphylococcal activity on five strains of *S. aureus* Meti-R showed the most vigorous activity of 70% aqueous ethanol compared to that of aqueous extract from stem bark [24].

Interestingly, together with antibacterial properties, the barks of *T. macroptera* could attenuate bacterial pathogenicity by interfering with the quorum sensing system on *Pseudomonas aeruginosa* PAO1 and by controlling the biofilm formation [25]. Ellagic acid was linked to this activity.

Finally, no toxicity has been described for *T. macroptera* (70% ethanol extract from barks), classifying its uses as traditional medicine as safe according to the OECD classification (DL_{50} >5000 mg/kg bm) [26,27]. Moreover, anti-hepatotoxicity of roots ethanol extract was reported by Yakubu et al. [28].

In 2015, a review of the genus Terminalia's chemical and biological studies had referenced 155 natural compounds in the different botanical parts of the plants. This literature survey revealed that genus *Terminalia* is a rich source of tannins and pseudotannins, including gallic acid and its simple gallate esters, chebulic and non-chebulic ellagitannins, ellagic acid derivatives, and ellagic acid glycosides. Phenolic acids, flavonoids, triterpenes, and triterpene glycosides are also present in high amounts and few lignan and lignan derivatives. Few of them have been described in *T. macroptera* through three studies [14,16,17].

Even though studies have been done on bioactivities and the chemical composition of *Terminalia macroptera* (leaves, root and stem barks), none were undertaken on heartwood.

Thus, in our course to discovering active compounds to fight infectious diseases and, more specifically, antimicrobial-resistant microorganisms and improve the qualitative analysis of a traditionally used plant, the present study deals with the phytochemical analysis of heartwood extracts of *T. macroptera* Guill. & Perr. using online characterization with UHPLC-DAD-ESI/qTOF-MS technique combined with antimicrobial assays on *Staphylococcus aureus* SA1199 (wild type) and a mutant SA1199B over-expressing NorA efflux pumps and resisting to fluoroquinolone.

2. Experimental

2.1. UHPLC-DAD-ESI/QTOF analysis

UHPLC-DAD-ESI/QTOF analysis was performed on an Agilent Infinity 1290 system (Agilent Technologies) coupled to a UV/vis DAD detector and to a QTOF 6530 (Agilent Technologies) detector.
controlled by MassHunter software (Agilent Technologies). Metabolite separation was carried out on a Poroshell 120 EC-C18 column (100 mm × 3.0 mm, 2.7 μm). The ESI source was optimized as follows: positive and negative ionization mode in auto-MSMS, scan spectra from m/z 100 to 2000, capillary voltage 3.5 kV, fragmentor 120 V, and fixed collision-induced dissociation (CID) energy at 20 eV. Nitrogen was used as the nebulizing gas with a flow rate of 10 L/min and a temperature of 300°C at 40 psi.

2.2. Plant sample

Plant samples were collected in June 2009 in Abker region (13° North, 21° East, Ouaddai, Chad) and was identified by comparison with the herbarium of the Veterinary and Zootecchnic Research Laboratory (LRVZ) of N’Djaména, the Ecological Museum of the Millennium (EMM) and the National Herbarium of Yaoundé (Cameroon). A voucher specimen (n° AB_03) was deposited at the EMM (Yaoundé, Cameroon).

2.3. Extraction and isolation

Dried trunk heartwoods (1 kg) were ground and extracted three times with 100% methanol (MeOH) (1 L, 3 times). Extracts were combined, and the solvent was evaporated to give an initial methanolic extract (125 g). The residue (30 g) was suspended in water (500 mL) and successively partitioned against dichloromethane (CHCl₃) (3 times, 300 mL), acetate d’éthyle (EtOAc) (500 mL, 3 times), and finally butanol (BuOH) (500 mL, 3 times). Solvents were evaporated under reduced pressure, and extracts were kept at -20°C until analysis and testing.

EtOAc extract (4.3 g) was subjected to a Vacuum Liquid Chromatography (VLC) on silica gel (100-200 mesh) eluted with a gradient of Hexane-EtOAc-MeOH (from 100% hexane à 100% MeOH) (supplementary data) and led to 8 fractions (F1 to F8). F7 (247 mg) was then submitted to a Solid Phase Extraction (SPE) column on reverse phase (Lichroprep RP-18, 40-60 μm). Elution with a gradient of H₂O-MeOH (from H₂O 100% to MeOH 100%, the step of 10%, 50 ml per step) gave 10 fractions (F7.1 to F7.10). F7.2, F7.6, F7.7, and F7.8 were submitted to filtration on Sephadex LH-20 in MeOH (100%). F7.2 (19.5 mg) led finally to compound 1 (7.8 mg).

F7.6 (31 mg) led to compound 11 (19.5 mg) and compounds 6-9 in mixture. F7.7 (18 mg) led to compounds 9 to 13 in the mixture. F7.8 (30 mg) led to four fractions (F7.8.1-F7.8.4). F7.8.2 (23 mg) gave five fractions after an ultimate filtration on Sephadex LH20 in the same conditions. In F7.8.2.1 (4 mg) compounds 2-5, 12 were characterized (Figure 2).

UHPLC-DAD-ESI/TOF analysis was performed using the following gradient with 0.4% aqueous acetic acid (solvent A) and acetonitrile (solvent B): 0 min, 1% B; 3 min, 1% B; 13 min, 100% B; 17 min, 100% B and 20% water (solvent B): 0 min, 5 mg/mL, 1% B; 3 min, 1% B; 13 min, 100% B; 17 min, 100% B. Flow rate was 0.7 mL/min and 5.0 μL of the sample were injected (c 5 mg/mL). Chromatograms recorded at λ 280 nm were selected for compounds detection. Metabolite annotation was based on Ultraviolet (UV), High-resolution mass spectrometry (HRMS), Tandem mass spectrometry (MSMS spectra), and relative retention time (RTs). Data were compared with the literature.

Structures of compounds 1 and 11 were confirmed by Proton and Carbon nuclear magnetic resonance (¹H and ¹³C-NMR) analysis and compared with literature (supplementary data). A Bruker Avance 400 apparatus is used for NMR experiments acquisition. Samples are dissolved in CDCl₃.

Data processing was performed as follows. Briefly, the UHPLC-HRMS raw data were processed with Agilent MassHunter software for mass and UV signals extraction. Molecular formula prediction and compound annotation of significant features (m/z, UV) were compared with literature or an internal database.

2.4. Antimicrobial activities

Two standard strains were selected for their implications in the recurrence of nosocomial diseases in hospitals, i.e. SA 1199 as wild type and the mutant SA 1199B over-expressing NorA efflux pumps. Experiments were carried out in 96-wells microplates using the micro-dilution technique adapted from literature. Extracts and fractions were tested in triplicate at concentrations ranging from 2 mg/mL to 0.0039 mg/mL (for the first step) and from 0.0039 mg/mL to 0.075x10⁻⁴ mg/mL (for the most active extract) in dimethyl sulfoxide (DMSO) (final conc. of 2.5% in well). Briefly, 100 μL of bacterial suspension were added to the extracts (100 μL) diluted in DMSO and medium. Concentrations of Ciprofloxacin range from 16 to 0.03125 μg/mL. The plates were incubated at 37°C for 24 h. DMSO and sterile broth were used as negative controls. OD’s are measured with a Vitek analyzer at λ 600 nm. Only data for active extracts/fractions are shown.

2.5. Radical scavenging activity

Radical scavenging activity concerning the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was evaluated at room temperature using a solution of DPPH at 0.8 mg/mL in MeOH. Trolox was used as positive control. Five dilutions ½ (5 mg/mL), ¼ (2.5 mg/mL), 1/8 (1.25 mg/mL), 1/16 (0.625 mg/mL), 1/32 (0.3125 mg/mL) of each sample were prepared starting from 10 mg/mL. Analyses were carried out in triplicate. DO’s of samples were measured at λ 515 nm using a microplates Multiskan Ex (Thermo Electron Corp.) with the software Ascent Software 2.6; the reading is done every minute during 45 min.

Extracts activity was evaluated through the Pi value (percentage of inhibition), which was calculated...
according to the formula: \( \text{Pi} = (1 - (\text{DO}_{\text{sample}} / \text{DO}_{100})) \times 100 \), where \( \text{DO}_{\text{sample}} \) = absorbance of the sample with DPPH and \( \text{DO}_{100} \) = absorbance of the DPPH solution (100 %). IC\text{50} value defined the effective concentration of antioxidant necessary to decrease the initial DPPH concentration by 50% and was calculated from the results by linear regression \(^{31}\).

3. Results and Discussion

There were qualitative differences between extracts, and considering the chromatogram profiles, the antimicrobial and radical scavenging activities, EtOAc extract was further investigated. We then compared the contents of fractions from the partitioning steps. Radical scavenging activity evaluation showed that EtOAc and BuOH extracts are useful as the positive control Trolox (Table 1).

|                | DCM\(^a\) | EtOAc\(^a\) | BuOH\(^a\) | Trolox  |
|----------------|------------|-------------|------------|---------|
| IC\text{50} (µg/ml) | 0.542 ± 0.11 | 0.208 ± 0.09 | 0.210 ± 0.10 | 0.229 ± 0.07 |

\(^a\) DCM: dichloromethane extract, EtOAc extract: ethyl acetate extract, BuOH: butanol extract

Figure 2. UHPLC-DAD-ESI chromatograms of fractions F7.6, F7.7 and F7.8.2.1. Part A) UV chromatograms at \( \lambda \) 280 nm. Part B) ESI-MS (negative mode) chromatograms
Table 2. MIC values of extracts and fractions from *Terminalia macroptera* heartwood.

|                     | MIC µg/ml |
|---------------------|-----------|
|                     | EtOAc extract | F7.6 | Ciprofloxacin |
| *Staphylococcus aureus 1199* | 0.244 ± 0.06 | 250 ± 23.0 | 0.25 |
| *Staphylococcus aureus 1199B* | 0.975 ± 0.10 | 62.5 ± 8.5 | 4 |

The most active fraction as an antimicrobial is the ethyl acetate extract, which presents an equivalent activity on *S. aureus* 1199 than ciprofloxacin with a Minimal Inhibitory Concentration (MIC) of 0.24 µg/mL versus 0.25 µg/mL. But on the resistant strain (*S. aureus* 1199B), its MIC value was 0.975 µg/ml versus 4 µg/ml for ciprofloxacin. Compound 11 was purified from this active extract and exhibited MIC values of 0.25 and 0.0625 mg/mL against sensitive and resistant strain, respectively (Table 2). Even though this activity is less than the positive control, it is interesting to notice the resistant strain's strongest effect. The extract EtOAc is more potent than the purified compound.

Metabolite profiling of EtOAc extract was acquired in positive and negative ionization modes. Qualitative analysis by LC-DAD-MS allowed the putative annotation of 13 major peaks through HRMS and MS/MS fragmentation patterns (Figure 2).

These compounds are mostly found in the Combretaceae family or in the genus *Terminalia* (Figure 1). Among the annotated compounds, 7 are tannin derivatives (gallic acid, galloyl quinic acid, ellagic acid, ellagic acid 2,8-di-methyl ether, ellagic acid 2,3,7-tri-methyl ether, valeneic acid dilactone and its methyl ester), 4 triterpenoids (terminolic acid, 23-O-galloyl terminolic acid, 23-O-galloyl arjunolic acid, 23-O-galloyl arjungenin), 2 stilbenoids (4,4’-dihydroxy-Z-stilbene-O-rutinoside and 3,5,4’-dihydroxy-Z-stilbene-O-rutinoside) (Table 3, Figure 3).
Table 3. Annotation by UHPLC-DAD-ESI/QTOF of the main compounds in EtOAc extract and fractions of *T. marcoptera* heartwood.

| Peak n° | t_R (min) | UV (nm) | m/z (M - H)* | UV (nm) | m/z (M + H)* | Formula | Annotation | Ref |
|---------|-----------|---------|--------------|---------|--------------|---------|------------|-----|
| 1       | 1.747     | 215,270 | 169.0125     | 215,270 | 141.0155     | C_{7}H_{5}O_{5} | gallic acid | Internal database |
| 2       | 4.732     | 200sh254, 305sh, 372 | 469.0092 | 299.0090 | C_{21}H_{10}O_{13} | Valoneic acid dilactone | 35 |
| 3       | 5.025     | 214, 274 | 345.0604     | -       | -            | C_{14}H_{10}O_{5} | galloyl quinic acid | Internal database |
| 4       | 5.745     | 222sh, 254, 373 | 483.0269 | 299.0093 | C_{22}H_{12}O_{13} | methyl valoneic acid dilactone | 35 |
| 5       | 6.045     | 222sh, 254, 373 | 300.9982     | -       | -            | C_{14}H_{10}O_{5} | ellagic acid | 38 |
| 6       | 7.282     | 250, 305sh, 355sh, 368 | 329.0240     | -       | -            | C_{16}H_{18}O_{9} | 3,3’-di-O-methyl ellagic acid | 40 |
| 7       | 7.585     | 230, 255sh, 322 | -           | 555.8672 | 521.3384 | C_{20}H_{26}O_{9} | 4,4’-dihydroxy-Z-stilbene-O-rutinoside | 43 |
| 8       | 8.300     | 248, 288sh, 358sh, 372 | 343.0451 | 328.0187 | 331.0295 | C_{19}H_{12}O_{8} | 3,3’,4-tri-O-methyl ellagic acid | 41 |
| 9       | 8.537     | 216, 276 | -           | 639.3517 | 639.3316 | C_{27}H_{38}O_{9} | 23-O-galloyl arjungenin | 16, 18 |
| 10      | 8.540     | 216, 275 | 655.3441, 1312.718 | 679.3355 | 639.3415 | C_{37}H_{52}O_{10} | 23-O-galloyl terminolic acid Confirmed by NMR data | 16 |
| 11      | 8.764     | ---     | 503.3405     | 1007.5859 | 469.3228 | C_{30}H_{48}O_{6} | terminolic acid | 16 |
| 12      | 9.140     | 216, 276 | 655.3498     | 657.3625 | 679.3432 | C_{37}H_{52}O_{10} | 23-O-galloyl arjungenin | 16, 40 |

UHPLC-DAD-ESI/QTOF-MS: Ultrahigh-performance liquid chromatography with diode array detection coupled to electrospray ionization and quadrupole time-of-flight mass spectrometry.
Figure 3. Chemical structures of annotated compounds in EtOAc from T. macroptera heartwood
Compounds 1 and 11 were isolated, and their structure was confirmed based on their UV, MS, and NMR data as gallic acid and 23-O-galloyl terminolic acid (Figure 3 and 4, Table 4).

Table 4. $^{13}$C NMR data of compound 11 (CDCl$_3$, 300 MHz).

| No. | Cpd 11 $^{13}$C (ppm) | Li et al. 2002 $^{13}$C (ppm) |
|-----|----------------------|-----------------------------|
| 1   | 50.2                 | 49.9                        |
| 2   | 69.3                 | 68.4                        |
| 3   | 78.3                 | 78.0                        |
| 4   | 44.4                 | 43.8                        |
| 5   | 49.8                 | 49.5                        |
| 6   | 68.4                 | 67.5                        |
| 7   | 42.8                 | 41.1                        |
| 8   | 39.8                 | 39.1                        |
| 9   | 49.8                 | 48.9                        |
| 10  | 38.6                 | 38.0                        |
| 11  | 24.00                | 23.5                        |
| 12  | 123.6                | 122.5                       |
| 13  | 144.7                | 144.1                       |
| 14  | 43.4                 | 42.6                        |
| 15  | 28.7                 | 27.8                        |
| 16  | 24.6                 | 23.8                        |
| 17  | 47.7                 | 46.6                        |
| 18  | 42.8                 | 42.0                        |
| 19  | 47.2                 | 46.3                        |
| 20  | 31.6                 | 30.8                        |
| 21  | 34.9                 | 34.1                        |
| 22  | 33.8                 | 33.0                        |
| 23  | 67.1                 | 66.8                        |
| 24  | 15.0                 | 15.4                        |
| 25  | 18.9                 | 18.8                        |
| 26  | 18.8                 | 18.6                        |
| 27  | 26.3                 | 26.0                        |
| 28  | 181.0                | 180.1                       |
| 29  | 33.8                 | 33.1                        |
| 30  | 24.0                 | 23.6                        |
| 1'  | 121.5                | 121.3                       |
| 2'/6' | 109.9               | 109.9                       |
| 3'/5' | 146.6              | 147.6                       |
| 4'  | 139.8                | 140.9                       |
| 7'  | 168.2                | 167.1                       |
The oligomers of tannins (gallic and ellagic acid, 3,3'-di-O-methyl ellagic acid, 3,3',4-tri O-methyl ellagic acid) were already indexed in roots of *T. macroptera* and identified by HPLC-UV-SM in fruits of three other *Terminalia* species: *T. bellerica*, *T. chebula*, and *T. horrida*. Ellagic acid in barks of *T. arjuna*, in leaves and fruits of *T. bellerica* and *T. chebula*, and leaves and fruits of *T. muelleri*.

These compounds are also widely described for their antioxidant properties, which corroborates the antioxidant activity of the present ethyl acetate and butanolic extracts of heartwood (Table 1). Ellagic acid and its derivatives were also described for their antiviral, bactericidal, antitumoral, anti-inflammatory, chemoprotection, and anti-leishmaniose properties. Oligomers of ellagitannins, isolated from the roots of *T. macroptera*, were active against *Shigella dysenteriae* and *Campylobacter* sp. In 2018, Ouedraogo et al. reported ellagic acid activity and its derivatives against the quorum sensing and as inhibitors of biofilm formation in *Pseudomonas aeruginosa*.

Besides these very well-known compounds, valoneic acid dilactone (2) and its methyl derivative (4) were characterized. The pseudo-molecular ions (m/z 469.0092 and 483.0269) are in accordance with the formula C$_2$H$_7$O$_5$(H)$_3$ and C$_2$H$_7$O$_5$(H)$_2$. The fragment ions at m/z 299.0090 and 299.0093 indicate the loss of gallic acid subunit [M - H - 153] and compounds 7 and 8 were annotated as 4,4'-dihydroxy-Z-stilbene-O-rutinoside and 3,5,4'-trihydroxy-Z-stilbene-O-rutinoside (or resveratrol-3-O-β-rutinoside) respectively. The E-configuration was determined with the UV data. Resveratrol-3-O-β-rutinoside was isolated in *T. sericea* root barks.

Finally, four triterpenoids were detected and annotated as terminolic acid (12), 23-galloyl terminolic acid (11), 23-galloyl arjunolic acid (10), and 23-galloyl arjungenin (13). Compounds 10 and 11 isolated in barks of *T. macroptera* exhibited activity against *Caenorhabditis elegans* (MIC = 50 and 100 µg/mL, respectively). *Pseudomonas aeruginosa* (MIC = 2.5 µg/mL) and *Bacillus subtilis* (MIC = 5 µg/mL).

Compound 7 is newly reported, and compounds 2, 4 are new in *Terminalia* genus, and finally, 8, 9, 13 are described for the first time in *T. macroptera*. Most of these compounds are known for their antimicrobial activities and could be related to the effectiveness of the EtOAc extract of heartwood of *T. macroptera* Guill. & Perr.

### 4. Conclusion

In the context of the multidrug bacterial resistance, this study on heartwood extracts of *T. macroptera* showed that EtOAc extract has a better MIC on the resistant *S. aureus* 1199B than ciprofloxacin. The mechanism of action must be specified since *S. aureus* 1199B overexpressed efflux pumps. This extract is a better radical scavenger than the positive control (Trolox). The UHPLC-DAD-ESI/QTDF-MS analysis led to a qualitative profile. Thirteen compounds were annotated due to their correspondence with already known compounds. Galloyl quinic acid, 23-galloyl terminolic acid, 23-galloyl arjungenin, and resveratrol rutinoside are known in the genus *Terminalia* but are described for the first time in *T. macroptera*. 4,4'-dihydroxy-Z-stilbene-O-rutinoside, a new compound, should be purified to complete its identification.

These components are known to have suitable antimicrobial activities and could be related to the ethyl acetate extract activity. *T. macroptera* has been qualified as a safe medicinal plant, this study encourages its development as a qualified traditional medicine.

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