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Antibacterial Activity of Leaf Extracts from *Combretum micranthum* and *Guiera senegalensis* (Combretaceae)

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ABSTRACT

African ethnopharmacology relays on the use of plants as a treatment of a wide spectrum of pathologies. The collaboration with Gruppo Solidarietà Africa (GSA onlus) allowed the selection of plants specifically used in West Africa as a traditional remedy for a number of diseases. *Guiera senegalensis* and *Combretum micranthum* (Combretaceae) leaves were selected and studied for the presence of antibacterial compounds. Plant material was sequentially extracted with a series of five solvents with an increasing polarity. Antibacterial activity against *Escherichia coli* C1a and *Staphylococcus aureus* MSSA (Methicillin sensible *S. aureus*) (ATCC 25293) was determined for the crude extracts and more purified fractions by means of agar disk and well diffusion assays. Moreover a novel bioautographic method was developed to follow the antibacterial activity throughout the purification steps, at the same time allowing to assess the ongoing fractions purification efficacy. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the most purified and active products were evaluated at the end of the procedure. The ethanolic extract of *C. micranthum* (Cm4) and the toluene extract from *G. senegalensis* (Gs2) showed the most relevant antibacterial activity. Two purified fractions, indicated as Cm4-P and Gs2-P, have been isolated from the most active crude extracts and their antibacterial efficacy was further studied against a panel of nine clinically relevant bacterial strains. Cm4-P resulted effective against both Gram positive and Gram negative strains, whereas Gs2-P was found active only against the Gram positive strains at very low concentrations (particularly MIC of 18.7 µg mL⁻¹ and MBC of 75 µg mL⁻¹ against *C. difficile* and *S. aureus*).

Key words: Antibacterial, *C. micranthum*, *G. senegalensis*, gram-positive, gram-negative, bioautography, MIC, MBC

INTRODUCTION

In most of African countries, a large number of diseases are treated administering plant infusions to the patients. This herbal therapy is used on a traditional basis and bequeathed through generations. Ethnopharmacology is still used up today as a treatment for sickness conditions ranging from abdominal pains, conjunctivitis and diarrhoea, to sexually transmitted

infections (Tshikalange *et al.*, 2008) or malaria (Gronhaug *et al.*, 2008). These therapies are mainly obtained by means of water or water/alcohol extractions of barks, leaves or roots of a variety of plants (Van Vuuren and Naidoo, 2010). A number of the above mentioned illnesses are caused or accompanied by the presence of pathogenic microorganisms such as bacteria, protozoa and fungi and, on the basis of their use, it is conceivable that some plants employed in ethnopharmacological practices probably contain antibacterial substances that could meet medical interest. Actually the research of promptly available and not expensive drugs remains an important goal for the global healthcare.

Up to these days a large number of scientific reports has been addressed to study the antibacterial activity of vegetable extracts. Many plant-derived compounds were found to exert characteristic antimicrobial activities, the most relevant of which belong to the families of flavonoids, terpenoids, polyphenols and alkaloids.

On the basis of the ethnopharmacology literature, most of the active substances can be found in leaves, roots or barks of African plants (Cowan, 1999; Cushnie and Lamb, 2005; Martini and Eloff, 1998).

In particular, the research of new antibacterial compounds is extremely important to cope with the spreading of antibiotic resistance among even unrelated bacterial pathogens, such as *Enterococcus faecium*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Streptococcus pneumoniae*. Beside these clinically relevant strains, an increasing number of other bacterial pathogens has been recently found resistant to a number of commonly used antibiotics including Vancomycin, considered as one of the last resource for the treatment of resistant bacterial strains (Rice, 2009).

Plants belonging to the family of Combretaceae are probably the most frequently used in the African traditional medicine for their large diffusion and high biodiversity (Combretaceae consists of 18 genera among which *Combretum* and *Terminalia* are the predominant with 370 and 200 species, respectively). Concerning this plant family, bioactive compounds were prevalently found in the leaves and the barks. The antitumor agent Combretastatin was isolated from bark of *Combretum caffrum* and this molecule is probably one of the most indicative examples of such substances (Eloff *et al.*, 2005). However, as reported in the literature, extracts from plants of the Combretaceae family were also tested against different microorganisms (Kuate *et al.*, 2010). Antifungal activity has been reported by Masoko for *Terminalia* species and by Suleiman for many other different plants (Masoko and Eloff, 2005, 2006; Suleiman *et al.*, 2010). Antiprotozoal effect of *Combretum molle* was discovered by Asres *et al.* (2001). Ferrea further reported the activity of *Combretum micranthum* against herpes simplex virus (Ferrea *et al.*, 1993). Moreover, the wound healing properties of Combretaceae extracts was studied by Masoko *et al.* (2010). In the West Africa, decoctions of *Combretum micranthum* leaves are widely used as anti-inflammatory treatments and further employed as a general panacea in case of indigestion, constipation or nausea. It is not a coincidence that this family of plants has been selected by the Association for African Medicinal Plant Standards as one of the 50 most important sources for botany-pharmacology (Eloff *et al.*, 2008).

At the Hospital St. Jean de Dieu, Tanguietà (Benin, West Africa), the leave infusions of two species of Combretaceae, *Combretum micranthum* and *Guiera senegalensis*, are largely used by local healers in the treatment of infected wounds and as a remedy for dysentery, thus an antibacterial activity of some components of the extracts from these two plants is conceivable. As some of the authors are member of the non-profit organization Gruppo Solidarietà Africa (GSA onlus) and operate out of that area for medical support, the dried leaves of the mentioned plants, along with botanic specimen certifications, could be provided.

In this study, it is reported the extraction of leaves of *Combretum micranthum* and of *Guiera senegalensis* with a sequence of solvents featuring an increasing polarity. The antibacterial efficacy of raw extracts was then tested against *E. coli* and *S. aureus*, considered as models of Gram negative and Gram positive strains, respectively. Partial purification of the most active fraction of each plant afforded new fractions whose antibacterial efficacy was further tested against a panel of clinically relevant bacteria. Their extent of efficacy was determined both in solid growth media and in solution. Disk and agar well diffusion assays or Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) test were also used.

MATERIALS AND METHODS

General: Analytical pure solvents, cyclohexane, toluene, acetone, diethylether (Et₂O) and ethanol (EtOH), purchased from Sigma-Aldrich, were used in the extraction/purification procedures; deionised water was used as solvent for the last step of extraction.

Chromatographic separations were performed on silica gel (Fluka, 0.063-0.2 mm size) and the thin layer chromatography analyses were developed on aluminium coated or glass coated Fluka silica gel/TLC cards with fluorescent indicator 254 nm (length 10 cm, thickness 0.2 mm). The Retention factors (R_f) are reported as fraction of the distance covered by the substance under studying with respect to the eluant front. Analytical grade (Sigma-Aldrich) dichloromethane, methanol (MeOH) and acetic acid (AcOH) were used for column chromatography and TLC development.

A Nicolet AVATAR FT-IR 360 instrument was used to record the infrared (IR) spectra of samples dispersed into KBr disks.

Atomic absorption analyses were performed with a Thermo-Electron SOLAAR series M instrument.

HPLC analyses were performed on an Agilent 1100 series instrument equipped with a diode array UV-Vis detector and fitted with a Supelco Ascentis C18 (25×4.6×5) or a Phenomenex Kinetex HILIC (15×4.6×2.6) column. The HPLC eluants were pure grade solvents (Sigma-Aldrich Chromasolv) and the flux was set at 0.5 mL min⁻¹ in each analyses.

Dry leaves extraction procedures: Whole leaves of *C. micranthum* and *G. senegalensis*, furnished by the botany section of the St. Jean de Dieu Hospital of Tanguietà (Benin), were collected in different periods (from June 2009 to September 2011), immediately dried in a local drying room at 40°C and finally sent to Varese (Italy).

Dried whole leaves (100 g) were manually minced, poured in a 2.5 L brown glass bottle and treated, under gentle mechanical stirring, with 600 mL of cyclohexane, the first and less polar solvent. After 24 h the leaves were separated from the solvent by filtration on a large Buckner funnel and the solvent was evaporated to dryness under vacuum by means of a rotating evaporator, thus affording the first raw extract, indicated as Gs1 and Cm1 for *Guiera* and *Combretum*, respectively. The extraction procedure was repeated, as described for cyclohexane, with all the other solvents of increasing polarity, i.e., toluene, acetone, EtOH and water in this order. After the extraction step the organic solvents were evaporated to dryness as described above whereas the aqueous mixture (the final extract) was frozen at -80°C and freeze-dried. Solid raw material was recovered evaporating each solvent affording *C. micranthum* (Cm 1-5) and *G. senegalensis* (Gs1-5) fractions (Table 1). Solutions at known concentration were prepared for each extract in a suitable solvent to be used for the antibacterial activity test.

Table 1: Weights, acronyms and antibacterial activities of the raw extracts (agar disk diffusion assay)

Solvents	<i>G. senegalensis</i> dry extracts (g)	Inhibition halos (mm $\varnothing$ halo - $\varnothing$ disk) for <i>S. aureus</i>	<i>C. micranthum</i> dry extracts (g)	Inhibition halos (mm $\varnothing$ halo - $\varnothing$ disk) for <i>S. aureus</i>
Cyclohexane	2.2 (Gs1)	0	1.5 (Cm1)	<4
Toluene	1.5 (Gs2)	5	0.5 (Cm2)	<4
Acetone	2.5 (Gs3)	0	1.4 (Cm3)	<4
Ethanol	5.6 (Gs4)	4	6.7 (Cm4)	5
Water (water)	6.6 (Gs5)	<4	6.0 (Cm5)	<4
Total weight	18.4		16.1	

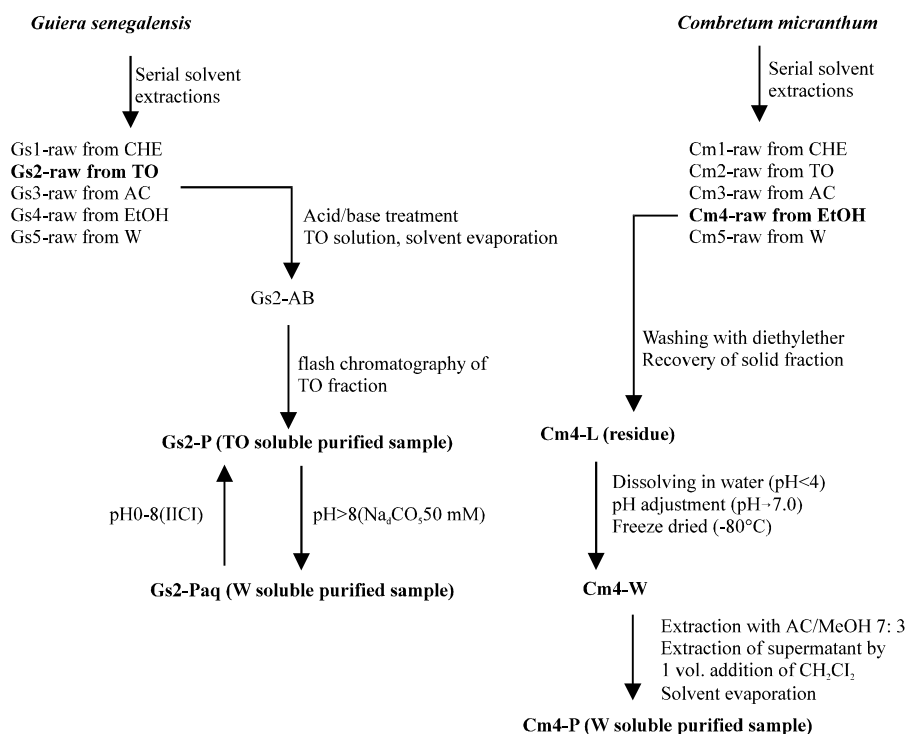


Fig. 1: Pathways to the partial purification of Gs2 and Cm4 extracts. The fractions used against the panel of bacteria reported in Table 2 are highlighted with bold character

Partial purification of crude extracts: The most active fractions from both *G. senegalensis* (Gs2) and *C. micranthum* (Cm4), identified following antimicrobial assays as described later on, were partially purified as reported in Fig. 1.

The toluene fraction from *Guiera* (Gs2, 1.5 g) was treated with an aqueous basic solution (pH>8.5) obtaining a reddish aqueous phase and a greenish organic phase. This last one was separated and discarded as devoid of any antibacterial activity; whereas the aqueous phase was acidified to pH 5.0 and then extracted with dichloromethane. The organic phase was dried (Na_2SO_4) and distilled off, under vacuum and gentle heating, producing a green solid product. This last was further purified by column flash-chromatography (SiO_2 , dichloromethane+0.4% AcOH) yielding 40 mg of a product whose purity was controlled by means of HPLC (column CPS Analytica TC-C18 5 μm -150 $\times$ 4.60 mm, eluant: solution A: CH_3CN 0.1% TFA, solution B: CH_2Cl_2 ; hold 70% B for 5 min,

linear gradient with 95% B at 10 min, flux 0.5 mL min⁻¹). The antibacterial activity of the Gs2-P fraction was tested either as its hydrophobic acidic form or as the water soluble form obtained at pH>8.5 (Gs2-Paq).

The active fraction from *Combretum* (Cm4, 6.8 g), isolated as greenish solid following EtOH evaporation, underwent a first purification step washing the solid four times with 50 mL Et₂O thus eliminating the residual lipophilic pigments. The residue was subsequently dissolved in the minimum amount of water, affording an acid aqueous solution (3<pH<4). This solution was neutralized via the careful addition of a few drops of NaOH (0.5 M) under the pH-meter control, thus increasing to 7.0 the pH value. Throughout the neutralization process the formation of an abundant precipitate was observed. This last one was separated by centrifugation and discarded as devoid of antibacterial activity whereas the water solution was frozen at -80°C and freeze-dried affording 3.6 g of a pale yellow solid (about 53% of the ethanolic raw extract). This batch went through a second purification step treating the solid with 100 mL of acetone/MeOH: 7/3 mixture. The insoluble material was eliminated by means of centrifugation and the supernatant was further treated with 100 mL of dichloromethane thus producing a new precipitate. The solid was again eliminated and the solution evaporated to dryness, affording 1.3 g of a highly hygroscopic, whitish solid (Cm4-P). TLC (SiO₂, CHCl₃/MeOH-8/2, with 1% acetic acid) showed the presence of one main product and that one of three other secondary compounds (R_f = 0.34 (main product), 0.5, 0.58 and 0.67, respectively).

Bacterial strains: The initial screening of the antibacterial activity of the crude fractions has been carried out against two tester strains, the Gram positive *S. aureus* MSSA (ATCC 29213) and the Gram negative *E. coli* C1a. Both strains were grown in LB at 37°C.

The efficacy of the purified fractions Cm4-P, Gs2-P and Gs2-Paq was evaluated, besides *S. aureus* and *E. coli*, on a panel of pathogen bacteria: *S. xylosus* (ATCC 29971), *Streptococcus pyogenes* (ATCC 19615), *Neisseria gonorrhoeae* (ATCC 49226), *Streptococcus pneumoniae* (ATCC 6305), *Haemophilus influenzae* (ATCC 49247), *Pseudomonas aeruginosa* (PAO1) and *Clostridium difficile* (ATCC 9689). All strain specifications can be obtained at <http://www.lgcstandards-atcc.org/>.

The culture media employed were: Agar chocolate (Sigma) for *N. gonorrhoeae* and *H. influenzae*; Agar blood (Sigma) for *S. pneumoniae* and *S. pyogenes*; Braziers agar medium (Sigma) for *C. difficile*. LB agar medium was prepared for *S. aureus*, *P. aeruginosa*, *S. xylosus* and *E. coli*.

Most of the bacteria were incubated at 37°C for 48 h, exclusive of *E. coli* C1a and *S. xylosus* that were incubated for a shorter time (24 h). All liquid cultures were incubated under shaking (350 rpm). *C. difficile* was incubated in an anaerobic growth chamber at Legnano Civil Hospital (Legnano, IT).

Growth media and buffers

LB growth medium: Triptone (OXOID) 10 g, Yeast Extract (OXOID) 5 g, NaCl (OXOID) 5 g, in 1 L of deionised H₂O.

Phosphate buffer (PB): KH₂PO₄ 6.8 g, K₂HPO₄ 8.7 g, in 1 L of deionised H₂O.

Antimicrobial activity assays

Bioautography method: A revised antibiogram (bioautography system) was developed to identify the presence of active components in the extracts. This method was developed as a modification of the contact TLC bioautographic method described by Choma and Grzelak (2011), to suit our specific purposes.

Initially each raw material has been carefully studied for its chromatographic behaviour on silica TLC plates following a variety of developments with different solvent mixtures. After the elution, the presence of organic compounds was detected displaying the plates under UV lamp (at 254 or 356 nm) or staining the TLC in a chamber of iodine vapour or, alternatively, spraying the plates with an oxidising reagent $[(\text{NH}_4)_6\text{MoO}_4, \text{Ce}(\text{SO}_4)_2, \text{H}_2\text{SO}_4, \text{H}_2\text{O}]$ followed by heating.

The TLC condition, resulting in the best separation of the components included in the studied fraction, was then applied on a 2 cm wide and 10 cm long TLC plate, all width loaded with approximately 50 μL of the fraction solution. After elution, the TLC plates were dried with an air flux and cut in the middle on their length; the two parts were then laid on LB agar plates, previously inoculated with the tester strains, at a distance of about 0.5 cm juxtaposing the cut edges and with the silica side put in contact with the culture medium. After appropriate incubation time at 37°C, the formation of inhibition zones between the two TLC halves could be observed, precisely indicating the presence of antibacterial activity corresponding to the R_f of particular components.

To verify the efficacy of this method, a commercial antibiotic (Augmentin®) was tested. A solution of this drug was prepared dissolving 10 mg in distilled water at a concentration of 2 mg mL^{-1} , then 50 μL (containing 58 μg of Amoxicillin and 8 μg of Clavulanic acid) were loaded on the TLC and developed with MeOH/dichloromethane: 8/2.

Agar diffusion assays: The agar disk diffusion method, as indicated by Clinical and Laboratory Standards Institute (NCCLS, 2006), was modified to observe the antimicrobial activity of both the crude extracts and of the purified fractions against some bacterial strains. For each strain, 4-5 isolated colonies were collected and resuspended in 5 mL of PB then inoculated on the appropriate solid growth medium by means a sterile cotton swab.

Filter paper disks (BBL™ TAXO™ BD) with a diameter of 13 mm were loaded with the appropriate volume of the solution of the extract under study at a known concentration. Repeated additions of 100-150 μL , up to a maximum of 500 μL , were made. The crude extracts from the first three solvents (cyclohexane, toluene and acetone) were dried, weighted and dissolved in dichloromethane, a suitable, volatile solvent. The solid residues recovered from the polar solvents (EtOH and water) were weighted and dissolved in a known volume of the corresponding extraction solvent. Cm4-L, Cm4-W, Cm4-P were used as aqueous samples while Gs2-P, totally insoluble in neutral aqueous phase, was used as dichloromethane solution. Alternatively, 4 mg of Gs2-P were solubilised in 1 mL of 50 mM Na_2CO_3 basic aqueous solution, affording the fraction Gs2-Paq. After the loading step, the disks were dried at constant weight under a gentle stream of warm air and placed on the culture plates. Reference disks were loaded with equivalent volume of dichloromethane or EtOH to assess the conceivable toxicity of residual solvent still eventually present.

The plates were incubated at 37°C for the appropriate time required by each microorganism. The antibacterial effect was estimated measuring the growth inhibition halo. An accurate evaluation of the inhibition halo could be achieved by means of a specially issued photo-camera, fitted with magnifying lens. The exact measure of the extent of the antibacterial effect was made

with the assistance of a photographic imaging elaboration software (GIMP). The data, expressed in mm, were obtained measuring two orthogonal diameters of the growth inhibition zone, then subtracting the diameter of the disk to the average value.

Agar well diffusion assays were performed with Cm4-P and Gs2-Paq against the same panel of bacterial strains used in the disk diffusion assay. Cm4-P solution was prepared dissolving the solid sample to a final concentration of $35000 \mu\text{g mL}^{-1}$ in PB, whereas the Gs2-Paq ($4000 \mu\text{g mL}^{-1}$) basic solution was obtained as described above. Plates were prepared and inoculated with the desired strain. Wells (about 6 mm diameter) were made carving the inoculated agar with the top end of a sterile Pasteur pipette, afterwards $20 \mu\text{L}$ ($700 \mu\text{g}$) of the Cm4-P and $40 \mu\text{L}$ ($160 \mu\text{g}$) of the Gs2-Paq solutions were poured inside the wells. The plates were appropriately incubated and the antibacterial activity was estimated measuring the inhibition haloes as previously reported.

Determination of MIC and $\text{MBC}_{99.9\%}$ values of the Cm4-P and Gs2-Paq fractions: The Minimal Inhibitory Concentration (MIC) and Minimal 99.9% Bactericidal Concentration ($\text{MBC}_{99.9\%}$) of each fraction were established according to the official CLSI (Clinical and Laboratory Standards Institute) protocol, against a panel of clinically relevant bacterial strains. A 96-wells microplate was loaded with $10 \mu\text{L}$ of a twofold dilution of a $5000 \mu\text{g mL}^{-1}$ aqueous mother solution of Cm4-P up to $2.4 \mu\text{g mL}^{-1}$, whereas Gs2-P was tested starting from an initial concentration of $1200\text{-}5.8 \mu\text{g mL}^{-1}$. Each well was then inoculated with $90 \mu\text{L}$ of 10^5 CFU mL^{-1} MH broth suspension of the appropriate bacterial culture. Controls were also set up loading the wells with the sterile growth medium or the plant extract solution or the bacterial inocula. Each test was repeated three times. The lowest extract concentration causing no visible growth was considered as MIC, whereas the lowest concentration that caused the death of 99.9% of the cultures, determined by viable count technique, was considered as the minimal bactericidal concentration ($\text{MBC}_{99.9\%}$).

Flavonoids and polyphenols detection: The shinoda color assay: A few magnesium turnings were added to an ethanol solution of the samples ($100 \mu\text{g mL}^{-1}$) then few drops of concentrated hydrochloric acid were added. After this treatment the onset of pink to dark red colours indicates the presence of various classes of flavonoids. In particular a colour ranging from orange to red indicates flavones, red to crimson indicates flavonoids, crimson to magenta indicates flavanones (Yisa, 2009).

RESULTS

Preparation and antimicrobial activity of crude extracts: The extraction of 100 g of dried leaves of *C. micranthum* or *G. senegalensis* with a sequence of solvents yielded comparable amount of the whole raw material. In Table 1 the list of all the extracts obtained from the two plants with acronyms and weights are reported.

Agar disk diffusion method ($1000 \mu\text{g}$ loaded) indicated that an effective inhibitory activity was observed for some of the extracts against *S. aureus*, whereas none of the isolated raw samples was active against *E. coli*. Control study disks that were loaded only with the solvents and subsequently air dried did not show toxicity, indicating that residual traces of solvent, possibly present, were not toxic to bacteria.

In the case of *G. senegalensis* the most active fraction was the lipophilic mixture obtained extracting the leaves with toluene (Gs2) which gave an inhibition zone of 5 mm. On the other hand the highest antibacterial efficacy for *C. micranthum* was found in the fraction recovered following evaporation of the polar solvent EtOH (Cm4) which gave a 5 mm halo (Table 1).

Bioautography method was applied on the raw materials as well as on the fraction isolated after each purification step. It allowed the identification of active compounds after each extraction/purification step even though present in a complex mixture. One example of bioautography of the raw fraction isolated from *G. senegalensis* (Gs2) against *S. aureus* MSSA is reported in Fig. 2a.

The reliability of this method could be confirmed evaluating the antibacterial effect produced by the commercial drug Augmentin® against *S. aureus* which as expected, gave a well evident, large inhibition zone (Fig. 2b).

Partial purification of Cm4 and Gs2 raw material and some chemico-physical peculiarities of the final isolated fractions: The most effective raw materials of both plants were purified following the pathways reported in Fig. 1.

Toluene extraction of 100 g of *G. senegalensis* dry leaves yielded 1.5 g of a green solid material (Gs2) that was purified following aqueous-organic phase repartition under controlled pH. The product obtained by extraction of the acidic aqueous phase with dichloromethane was further purified through column flash-chromatography affording 40 mg of a green solid product (Gs2-P, about 2.6% of the raw material) showing only one spot on TLC with $R_f = 0.39$ (SiO_2 , $\text{CH}_2\text{Cl}_2/\text{MeOH}$: 97/3), thus it can be considered as a single compound. This green substance is perfectly soluble in dichloromethane and no repartition was observed in slightly acidic (pH 5.0) or neutral aqueous phase. On the other hand, a complete solubility in a basic aqueous phase (pH>8.5) was observed as long with a colour change from green to dark reddish hue (Gs2-Paq). The pH dependent solubility indicates the presence of at least one acid moiety on the molecule. The presence of either a carboxylic acid or an aromatic hydroxyl group (phenolic group) can be considered as both these chemical groups are commonly present in the natural products. The green solid compound does not melt up to 300°C, however a peculiar behaviour was observed in the course of this assay. Above 200°C the product colour turns from green to white but it comes back to green on cooling. Quite

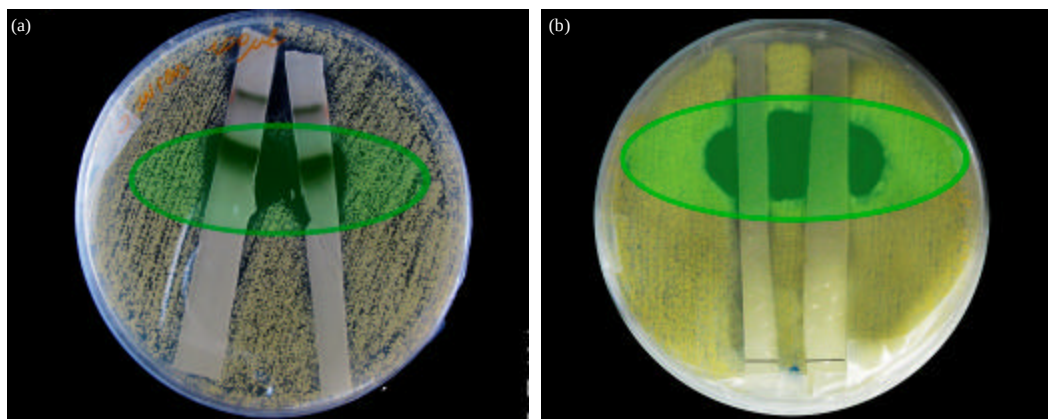


Fig. 2(a-b): Example of bioautography (COBACHI) on *S. aureus* MSSA of (a) Gs2-AB fraction (125 µg) after elution on silica gel TLC with dichloromethane/MeOH: 97/3 and (b) Commercial antibiotic drug Augmentin® (Amoxycillin/Clavulanic acid); 100 µg of the whole tablet were loaded on the TLC and developed with MeOH/dichloromethane: 8/2

surprisingly the TLC and UV-Vis analyses performed after such a severe heating indicate that no structure alteration has occurred to the sample. Actually the absorbance profile was unchanged and the presence of only one single spot, showing the same R_f of the original compound, was detectable. With the aim to find an explanation for this peculiar behaviour, atomic absorption analyses were also performed to identify the possible presence of any metal complexes, however none of the following metals of the 3rd period (Co, Cr, Cu, Fe, Mn, Ni, Zn) could be detected.

As far as *Combretum* is concerned, the highest activity was observed for the raw material recovered with EtOH extraction (Cm4, 6.7 g). From this fraction the partially purified Cm4-P was isolated after several purification steps. As it was found that the Cm4 aqueous solution gave an acidic pH (about 3.5) it was adjusted to 7.0 following the addition of few drops of a NaOH solution. This pH modification produced a copious precipitate then discarded as devoid of activity whereas the aqueous phase was frozen at -80°C and freeze-dried. The solid thus obtained was washed with several organic solvents and finally brought into solution with an organic solvent mixture (dichloromethane/acetone/MeOH: 5/3.5/1.5), thus affording the Cm4-P fraction (Fig. 1). This fraction was characterized by the presence of 4 compounds featuring different Running factors (R_f) on TLC (R_f = 0.34, 0.5, 0.58 and 0.67; SiO_2 , CHCl_3 -MeOH: 8/2, with 0.1% of AcOH), however the compound characterized by the lowest R_f value was largely the most abundant. All these compounds are scarcely detectable on TLC under UV lamp irradiating either at 254 or 365 nm wavelengths. This result is particularly indicative of the absence of a particularly extended conjugated π electronic system and the presence of only isolated phenyl rings can be envisaged in their structure. Moreover these compounds were found very well detectable by means of I_2 vapour, thus the presence of unsaturated organic molecules is reasonable. Despite the considerable difference in the running factors of the 4 compounds, several attempts to carry out chromatographic separation did not allow the isolation of a single product. This peculiar behavior could be probably due to a decomposition of these compounds, as witnessed by the formation of dark brown products that stuck on the top of the silica gel column in the course of this purification procedure. The Cm4-P fraction is a pale yellow, highly hygroscopic solid, with an undefined melting point as it begins to shrink at about 60°C then assuming an amorphous aspect at about 140°C , along with the formation of a dark brown product that does not change its aspect on increasing the temperature. IR spectroscopy shows the presence of an intense signal in the range $3400\text{--}3300\text{ cm}^{-1}$ that is indicative of the presence of hydroxyls. Consequently a polyphenolic structure characteristic of the flavonoid family might be hypothesised for this compound. The presence of flavonoids as the main components of the extract is also supported by evidence in the Shinoda test: Cm4-P developed a reddish colour thus confirming the presence of a flavone and/or a flavanone structures.

Antimicrobial activity of *C. micranthum* and *G. senegalensis* extracts (agar diffusion assays)

Agar diffusion assays: The crude extracts antibacterial activity data were obtained by means of agar disk diffusion assay against the two tester strains (*E. coli* and *S. aureus*) and are reported in Table 1. Among the fractions isolated with solvents of different polarity, in the case of *Combretum*, the most conspicuous and most active fraction was obtained extracting the leaves with EtOH (Cm4), although active fractions were also found in the extracts obtained with the other solvents. Differently, in the case of *Guiera* the low polar toluene was found the solvent affording the most active fraction (Gs2), albeit from the polar solvents active fractions have been also isolated.

The purified fractions were also evaluated by means of agar diffusion assays against a panel of bacterial pathogens.

When disk diffusion assay was used (Table 2), an overall higher activity of the purified fractions with respect to the raw material was observed. In fact the Cm4-L, Cm4-W and Cm4-P fractions from *C. micranthum* were found active against the Gram positive *S. aureus*, *C. difficile* and *S. xylosus*. Furthermore Cm4-P showed a partial activity also against the Gram negative strains *E. coli* and *P. aeruginosa*, although with a lower efficacy as compared with the effect exerted against the Gram positive bacteria.

As far as Gs2-P is concerned, a promising activity was found against *S. aureus* MSSA and *S. xylosus* while no effect was detected against the other bacterial strains. On the basis of the disk diffusion assays, Gs2-Paq was devoid of activity, however a possible effect of retention of the organic substance on the study disk could be considered. To overcome the possible retention of antibacterial compounds on the cellulose disks used for the assays, well diffusion assays were performed. By means of this last method the antibacterial activity of the fractions was evaluated in direct contact with the culture medium (Table 3). Cm4-P and Gs2-Paq were then tested against the same panel of microorganisms used in the disk diffusion assay, although lower concentrations were used with both compounds (700 and 160 $\mu\text{g mL}^{-1}$, respectively). A 50 mM Na_2CO_3 solution, used to prepare Gs2-Paq, was also tested as the negative control. As far Cm4-P was concerned the halos were comparable with those obtained in the case of disk diffusion, whereas Gs2-Paq gave measurable halos against *C. difficile*, *S. aureus* and *S. xylosus* that were not observed in the case of the disk diffusion previously used. Unfortunately the absence of activity of Gs2-Paq against Gram negative bacteria was confirmed also with the agar well diffusion test.

Table 2: Inhibition halos (mm $\varnothing$ halo – $\varnothing$ disk) of selected fractions (agar disk diffusion assay)

Bacterial strain	Partially purified		Purified		
	Cm4-L (1000 μg)	Cm4-W (1000 μg)	Cm4-P (1000 μg)	Gs2-P (1000 μg)	Gs2-Paq (1000 μg)
<i>S. aureus</i> MSSA ^a	7	11	15	18	<4
<i>C. difficile</i> ^a	8	13	14	<4	<4
<i>S. xylosus</i> ^a	8	10	12	16	<4
<i>S. pyogenes</i> ^a	<4	<4	<4	4	<4
<i>S. pneumoniae</i> ^a	<4	<4	<4	<4	<4
<i>N. gonorrhoeae</i> ^b	<4	<4	<4	<4	<4
<i>H. influenzae</i> ^b	<4	<4	<4	<4	<4
<i>E. coli</i> ^b	<4	<4	6	<4	<4
<i>P. aeruginosa</i> ^b	<4	<4	5	<4	<4

Table 3: Inhibition halos (mm $\varnothing$ halo – $\varnothing$ well) of selected fractions as observed in the agar well diffusion assay

Bacterial strain	Cm4-P (700 $\mu\text{g well}^{-1}$)	Cm4-P (1000 $\mu\text{g disk}^{-1}$)*	Gs2-Paq (160 $\mu\text{g well}^{-1}$)	Gs2-Paq (1000 $\mu\text{g disk}^{-1}$)*
<i>S. aureus</i> MSSA ^a	16	15	6	<4
<i>C. difficile</i> ^a	18	14	8	<4
<i>S. xylosus</i> ^a	16	12	6	<4
<i>S. pyogenes</i> ^a	<4	<4	<4	<4
<i>S. pneumoniae</i> ^a	7	5	<4	<4
<i>N. gonorrhoeae</i> ^b	<4	<4	<4	<4
<i>H. influenzae</i> ^b	<4	<4	<4	<4
<i>E. coli</i> ^b	4	<4	<4	<4
<i>P. aeruginosa</i> ^b	<4	<4	<4	<4

Table 4: MIC and MBC values obtained for the most purified fractions

Bacterial strain	Cm4-P		Gs2-Paq	
	MIC ($\mu\text{g mL}^{-1}$)	MBC ($\mu\text{g mL}^{-1}$)	MIC ($\mu\text{g mL}^{-1}$)	MBC ($\mu\text{g mL}^{-1}$)
<i>S. aureus</i> MSSA ^a	625	1250	18.7	75.0
<i>S. xylosus</i> ^a	625	1040	37.5	37.5
<i>C. difficile</i> ^a	156	312	18.7	750.0
<i>E. coli</i> ^b	1250	>1500	>1500.0	>1500.0
<i>P. aeruginosa</i> ^b	1250	>1500	>1500.0	>1500.0

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC): The MIC and the MBC of Cm4-P and Gs2-Paq were evaluated against the bacterial strains that were found more sensitive to the action of these extracts in the previous assays, namely *S. aureus* MSSA, *S. xylosus*, *C. difficile*, *E. coli* and *P. aeruginosa* (Table 4).

As expected, Cm4-P showed a good activity against *S. aureus* and *S. xylosus*, however *C. difficile* proved to be the most sensitive strain towards this extract (MIC = 156 $\mu\text{g mL}^{-1}$, MBC = 312 $\mu\text{g mL}^{-1}$) among the tested bacteria. In addition Cm4-P showed a partial activity also against the Gram negative testers, although at such concentrations (>1000 $\mu\text{g mL}^{-1}$) that cannot be considered of clinical interest.

Gs2-Paq was found extremely more active against all the Gram positive strains with respect to Cm4-P, as it showed MIC and MBC values in the range of a few tens of micrograms. On the other hand, once more no activity was observed against the Gram negative bacteria (Table 4).

DISCUSSION

The leaf, root and bark extracts of plants of the Combretaceae family have been studied for their antibacterial activity (Masoko and Eloff, 2005) due to the presence of a wide number of bioactive compounds in the literature, including flavonoids, stilbenoids (combretastatins), tannins (Cowan, 1999), polyphenols (Cushnie and Lamb, 2005) and terpenoids (Ntchatcho *et al.*, 2009) that were extracted from plants belonging to the Combretaceae family. These plants are composed of many different species among which *Molle*, *Erythrophyllum*, *Imberbe* and *Woodii* have been largely investigated (Martini *et al.*, 2004; Udoh *et al.*, 2012). On the other hand *C. micranthum* has not so deeply investigated so far. Beside the findings on its antiviral activity (Eloff *et al.*, 2008) and the anti-hyperglycaemic activity (Chika and Bello, 2010), only a few scientific reports, concerning the antibacterial activity of methanol and aqueous extracts of different parts of this plant, have appeared in the literature (Udoh *et al.*, 2012; Taura *et al.*, 2009; Karou *et al.*, 2005).

As far as *G. senegalensis* concerns, molecules with different chemical structures with respect to those found in *Combretum* were identified and guieranone or flavasperones can be enumerated among the active compounds which showed anti-diarrhoeic and anti-inflammation activities (Silva and Gomes, 2003; Mahmoud and Khalid, 1997). However, up to date, no antibacterial applications have been recognized for the extracts obtained from various vegetable materials of this plant.

The solvent extraction applied to dry and finely minced plant parts is the first procedure in the isolation of active molecules. In this regard, the choice of the solvents is particularly crucial as they can favour a selective isolation of different components. Actually the specific chemico-physical properties of low polar organic compounds address their solubility in organic solvents with low

dielectric constants, such as hexane, toluene and dichloromethane. On the other hand, the use of water or alcohols (EtOH and MeOH are the most frequently used) ensures the isolation of highly polar components from vegetable material (Eloff, 1998; Obeidat *et al.*, 2012).

These considerations suggest the optimization of leaves extraction by means of a sequence of solvents of increasing polarity (dielectric constant k), starting with the lowest polar solvents, cyclohexane and toluene ($k = 2.0$ and 2.4 , respectively), followed by acetone ($k = 21$) and then ethanol ($k = 24$). Water ($k = 80$) was used as the last solvent, also according to the procedure reported by Angeh *et al.* (2007).

The amount of extracted product was dependent on the solvent polarity, as greater quantity of substance (about 6-7% of the total leaves dry weight) was obtained with the more polar solvents while the solvents featuring lower polarity gave only 0.5-2.5% of the dry weight.

The screening of the crude extract efficacy was performed against two tester bacterial strains, *S. aureus* and *E. coli*, used as model for Gram positive and Gram negative bacteria, respectively. Despite the absence of activity against *E. coli*, some of the crude extracts from both *Guiera* (Gs2, Gs4 and Gs5) and *Combretum* (Cm4 and Cm5) showed a promising antimicrobial activity against *S. aureus*. The highest antibacterial efficacy was observed for the lipophilic *Guiera* extract Gs2, obtained with toluene and for the polar alcoholic extract (Cm4) of *Combretum* (Table 1). Repeated extractions on different batch of leaves of the two plants collected in different period from May 2010 to September 2012 gave comparable results as far it concerns the amount of material extracted as well as the activity found against the tester bacteria (data not shown).

The purification procedures of the most active crude extracts (Cm4 and Gs2) were designed taking into account the distinct chemico-physical properties of the two raw products as witnessed by their solubility in solvent with different polarity. In the case of Gs2 the first purification step relies on the solubility in organic solvents of its acidic form and on the repartition in an aqueous solution under basic conditions. Furthermore, a chromatographic purification step could be finally performed because of the extraordinary chemical stability of this compound.

Otherwise the purification of the *Combretum* extract (Cm4) relies on the poor solubility in aqueous phase at neutral pH of most of the undesired contaminating products so that can be easily eliminated by filtration or centrifugation. These solid precipitates were ineffective antibacterial compounds, however they could contain a product that is useful for the treatment of diabetes, recently described in a patent and whose structure contains a piperidine-flavan alkaloid (Simon *et al.*, 2011).

Cm4-P, isolated after lyophilization and solvent washing, seems to be still composed of about 4 substances, one of which is largely more abundant, as evidenced by TLC and HPLC analyses. Unfortunately further purification could not be achieved because of the instability of this product during the chromatographic process. Visible changes of this sample were observed on silica gel after a few minutes of elution as colored compounds strongly binding to the stationary phase have appeared, thus preventing the recovery of a purified active product.

After each single purification step, the identification of the portion maintaining the antibacterial activity was assessed both measuring the inhibition zones in the disk diffusion test and observing the bacterial growth inhibiting areas obtained with a modified bioautography (COBACHI). It is worth noting that this last method, based on TLC separation of the sample components, allowed the determination of the presence of one or more active compounds. The absence of inhibition zones around TLC spots clearly indicates that non active compounds are

present, thus giving a direct assessment about the composition of the analysed fraction. From these data the conditions for a possible purification by means of a column chromatography could be directly envisaged on the basis of the R_f values.

In some cases, 1 mL DMSO/H₂O 1:1 solution can be sprayed on the TLC silica-gel surface before the deposition on the agar-growth medium to facilitate the diffusion of low polar compounds in the aqueous growth medium.

All the purification steps here reported yielded more active fractions with respect to the raw materials, featuring inhibition halos about three times larger on *S. aureus* (compare results of Gs2 and Cm4 in Table 1 with Gs2-P and Cm4-P in Table 2). Moreover a partial activity of Cm4-P was also observed against *E. coli*, not detectable with any of the Cm4 raw fractions or with the partially purified compounds Cm4-W and Cm4-L.

The increased antibacterial efficacy of the purified fractions with respect to the raw materials, as observed for the fractions here reported, is not strictly consequent. Indeed it has been reported of higher antibacterial activity of unrefined extracts as compared to the single isolated antibacterial substance. This result has been interpreted by the authors considering a synergistic effects exerted by different components present in the original mixture (Lee *et al.*, 2005). Obviously, the higher efficacy of the purified fractions here reported clearly indicates that the purification steps described in this work enable the loss of non active components only.

The efficacy of Gs2-P and Cm4-P fractions, together with some other active intermediate isolated in the purification of ethanolic extract of *Combretum* (Cm4-L and Cm4-W) and the aqueous solution of purified *Guiera* sample (Gs2-Paq), were also tested against a panel of bacteria responsible for nosocomial infections by means of agar disk diffusion assays (Table 2). In general, as already observed for the crude extracts, the purified compounds were found more active against Gram positive than Gram negative bacteria. Among these last only *E. coli* and *P. aeruginosa* are partially sensitive to Cm4-P only (Table 2, column 3).

It is worth noting the high activity observed in the case of the Cm4-P fraction and of its precursors (Cm4-L and Cm4-W) against *C. difficile*, a well known pathogen in nosocomial infections. Gs2-P seems to be more efficient against *S. aureus* and *S. xylosus* strains than Cm4-P (Table 2, columns 4 and 3). Furthermore Gs2-P showed activity also against *S. Pyogenes* in the agar disk diffusion test, although in low extent.

The antibacterial effects observed are very interesting and may also meet clinical interest as some of the bacteria sensitive to the purified fractions are known to be hardly eradicated by the most common antibiotics, as in the case of *S. aureus* and *C. difficile* (Table 2).

None of the extracts from the two plants showed activity against the Gram negative *H. influenzae* and *N. gonorrhoeae*.

The results obtained with disk diffusion assays can be influenced by the retention of part of the organic matter on cellulose. This effect is particularly evident in the case of the water soluble Gs2-Paq fraction that does not migrate from the disk to the agar medium thus resulting inactive towards all the tested bacteria (Table 2). On the contrary, in the agar well diffusion assay (i.e., when the substance is in direct contact with the bacteria growing medium), this same fraction showed an interesting antibacterial effect against *S. aureus*, *C. difficile* and *S. xylosus*, as haloes of 6-8 mm were observed with only 160 µg of product whereas 1000 µg loaded on a disk did not gave any positive effect (Table 3, column 3 and 4).

Particularly, when antibacterial activity was evaluated by means of agar well diffusion (Obeidat *et al.*, 2012), an observable difference in the efficacy of Cm4-P was also measured as

700 $\mu\text{g well}^{-1}$ afforded haloes comparable or slightly higher with respect to those observed in the case of disk diffusion loaded with 1000 μg (Table 3). These results support an interpretation of a partial retention of the antibacterial compound on the study disk.

The different amount of Cm4-P and Gs2-Paq used in the disk and in the agar well diffusion test (1000 and 160 $\mu\text{g well}^{-1}$, respectively) is justified by the difficulty to solubilise the latter in an aqueous phase. Indeed the *Combretum* extract can be easily dissolved in water at high concentration (up to 40000 $\mu\text{g mL}^{-1}$), whereas Gs2-P can be only solubilised in the basic water solution at a maximum of 4000 $\mu\text{g mL}^{-1}$. Higher concentration could be obtained using more basic solution i.e., 100 mM Na_2CO_3 , however this alkaline solution resulted harmful for most of the bacterial strains tested (data not shown).

The MIC and MBC values of last purified fractions have been evaluated to better understand their antibacterial potential. As far it concerns Cm4-P, we found that the MIC values against Gram positive and Gram negative testers are just slightly different, with the exception of *C. difficile* that was found fairly the most sensitive. The MBC/MIC ratios for all the tested bacterial strains are >1 suggesting a bacteriostatic rather than a bactericidal effect (Karou *et al.*, 2005). Once again the efficacy of the Gs2-Paq fraction against the Gram positive bacteria was extremely higher with respect to Cm4-P; against *S. xylosus*, the effect of this fraction can be considered bactericidal as the MIC/MBC ratio equals to 1. Unfortunately, no activity could be observed against the Gram negative strains up to 1500 μg of substance used.

CONCLUSION

Among the active and partially purified fractions the product recovered in larger quantity was that one from *C. micranthum*, thus its practical application could be envisaged. Actually the purification of ethanol active fraction (Cm4) affording the final compound Cm4-P does not require chromatographic procedures as a simple pH adjustment of its aqueous solution and, eventually, the treatment of the residual solid with a mixture of organic solvents is required. It is interesting to observe that the antibacterial activity of Cm4-W appears not so much different from that one of Cm4-P, then the procedure of partial purification could be concluded at the step of the pH adjustment, i.e., a procedure that can be quite easily performed also in the absence of specific laboratory equipment. Regarding *G. senegalensis*, a poor amount of active compound can be isolated from the leaves after the purification procedures; however, an easy protocol has been here described to recover this compound. Despite the few tens of milligrams isolated from 100 g of dry leaves, the high and selective efficacy of Gs2-P against Gram positive bacteria, envisage clinical applications of this compound as possible outlooks.

A last comment concerns the new method set up for the identification of antibacterial activity (COBACHI). Despite of its simplicity, this method allows fast and unambiguous identification of the antibacterial component(s) in a mixture, thus its divulgation could be particularly interesting for the scientific community.

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