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In vitro anti-*Neisseria gonorrhoeae* activity of *Senna podocarpa* root extracts

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ABSTRACT

Senna podocarpa (Guill. & Perr.) Lock leaf and root are used in African countries to treat sexually transmitted diseases, namely gonorrhoea.

A bioguided fractionation study was performed to evaluate the antibacterial activity of a *S. podocarpa* root hydro-ethanol extract (Spr).

Spr, its major compounds and liquid–liquid partition fractions (Spr-1, diethyl ether; Spr-2, ethyl acetate; Spr-3, water) as well as chromatographically isolated main compounds of Spr-1, were tested against nine *Neisseria gonorrhoeae* reference and clinical strains, including strains with diminished susceptibility to penicillin, tetracycline, and ciprofloxacin. Spr showed anti-*N. gonorrhoeae* activity against all tested strains with minimum inhibitory concentrations (MIC) ranging from 100 to 400 mg/L.

The main compounds isolated from the most active fraction, Spr-1 (MIC: 50–100 mg/L) were identified as rhein (1), emodin (2), chrysophanol (3) and physcione (4) by LC-UV/DAD co-chromatography with reference standards. Rhein (MIC: 3.13 mg/L against all test strains) proved to be the most active of the isolates, while 2–4 and sennosides A and B (5–6), the major compounds of the total extract, were inactive in the range of tested concentrations (MIC > 75 mg/L).

Rhein was shown to be responsible for the anti-*N. gonorrhoeae* activity of *S. podocarpa* root and it can be used as a marker compound to assess the antibacterial activity of this medicinal plant.

1. Introduction

Gonorrhoea is one of the most common sexually transmitted infections in Africa (Kenyon et al., 2014). Previous studies have found that practitioners in Guinea-Bissau referred among others, *Senna podocarpa* (Guill. & Perr.) Lock (Leguminosae–Cesalpinioideae) root decoctions for the treatment of this disease (Diniz et al., 1996; Silva et al., 1997). Other plants have been shown to be equally important in inhibiting *Neisseria gonorrhoeae*, including isolate strains resistant to antibiotics (Cybulska et al., 2011).

N. gonorrhoeae has developed resistance to the majority of the recommended antibiotics used for its therapy. Thus, at present, third generation cephalosporins are mainly the drugs of choice to treat and control this infection (Tapsall, 2009). However, considering the prohibitively expensive treatment of gonorrhoea in most developing countries (Lewis et al., 2008), where resistance to the antibiotics is growing, herbal remedies are the only gonococci infection treatment accessible to rural populations (Silva et al., 2002).

S. podocarpa, formerly known as *Cassia podocarpa* Guill. & Perr., is a medicinal plant which leaves and roots are used in all West African countries for different purposes besides the treatment of sexually transmitted diseases (Adjanohoun et al., 1994). A screening for the antimicrobial activity of these plant extracts showed activity against *Campylobacter coli*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Shigella dysenteriae*, *Staphylococcus aureus*, *Streptococcus faecalis* and *Vibrio cholerae* strains (Silva et al., 1996). Furthermore, in a study on Guinea-Bissau's plants used to treat gonorrhoeae, hydro-ethanol extracts of *S. podocarpa* leaf and of *S. podocarpa* root showed an in vitro activity against

Abbreviations: Spr, *Senna podocarpa* root hydro-ethanol extract; Spr-1, diethyl ether liquid–liquid fraction of *S. podocarpa* root extract; Spr-2, ethyl acetate liquid–liquid fraction of *S. podocarpa* root extract; Spr-3, water liquid–liquid fraction of *S. podocarpa* root extract.

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N. gonorrhoeae strains with different susceptibilities to penicillin and tetracycline. In this study, the root extract proved to be more active than the leaf extract (Silva et al., 1997). In the present work, in order to confirm the activity of the *S. podocarpa* root against *N. gonorrhoeae* and identify the active constituents, a bioactivity-guided fractionation study of a 80% hydro-ethanol root extract (Spr) was performed. This approach included the testing of the antimicrobial activity (anti-*N. gonorrhoeae*) of either the total extract and its marker compounds as well as the major compounds obtained from the most active Spr liquid–liquid partition fraction. These major compounds were isolated and identified using usual chromatographic and spectroscopic techniques.

2. Materials and methods

2.1. Plant material

S. podocarpa roots were collected in the Contuboel Sector of Bafata Region in Guinea-Bissau. The plant material was identified according to a reference method (Resources Inventory Committee, 2015), using the distinctive anatomical characteristics described by Guillemin et al. (1830) and authenticated by Dr. Adélia Diniz of the Tropical Botanic Garden in Lisbon. Voucher specimens are preserved in LISC Herbarium of "Jardim Botânico Tropical" in Lisbon, Portugal, under the designation M.A. Diniz, 628.

2.2. Extract preparation, liquid–liquid fractionation

The *S. podocarpa* root hydro-ethanol extract (EtOH-H_2O, 80:20 V/V) (Spr) was prepared according to the methodology previously described (Silva et al., 1997) from 302 g of powdered plant material. A portion (17.5 g) of this extract was subsequently fractionated by sequential liquid–liquid partition with diethyl–ether (Spr-1), ethyl acetate (Spr-2) and water (Spr-3), according to the Charaux partition method modified by Paris and Nothis (Paris and Nothis, 1970).

The extract and fractions were evaporated to dryness under reduced pressure at 35 °C, yielding each fraction 4.4 g, 2.5 g and 10.6 g, respectively. About 100 mg of the dried extract and fractions were then dissolved in dimethyl sulfoxide (H_2O-DMSO, 90:10 V/V) to a final concentration of 100 mg mL^{-1} and the resulting solutions were used in the antibacterial studies.

All solvents were purchase from Merck and of pro-analysis or HPLC grade.

2.3. LC-UV/DAD analysis

Spr extract and fractions were analysed by LC-UV/DAD using a Waters Alliance instrument with a 2690 photodiode array detector. Separation was achieved by using a Nova-Pak 4 μm C18 column (150 × 3.9 mm I.D.) from Waters, equipped with a Sentry-Pak C18 pre-column. A MeOH-H_2O containing 0.05% TFA step-gradient was used (5:95–100:0 in 40 min). The flow rate was 0.9 mL/min. The UV chromatogram was recorded between 210 and 450 nm using the MaxPlot Waters Millenium software.

Sennoside A, sennoside B, rhein, emodin, chrysophanol and physcione from Extrasynthese were used as reference standards.

2.4. Isolation and identification of pure compounds from Spr-1

Isolation and purification of active compounds from Spr-1 fraction (3.0 g) were performed using silica gel 60 (100–200 mesh, Merck) preparative column chromatography using a gradient from 100% of hexane to 100 % of ethyl acetate. After TLC control using silica gel 60 F254 Ref. 5554 Merck, petroleum ether–ethyl acetate–formic acid (75:25:1 V/V) as mobile phase system and KOH 5% as spraying reagent, six major fractions were obtained, according to the chemical composition. Fractions 2, 4–6 were subsequently submitted to silica column chromatography using hexane-ethyl acetate 90:10 (V/V). The major compound of each fraction, compounds 1 (8 mg), 2 (5 mg), 3 (3 mg) and 4 (3.6 mg) were, respectively, purified by means of a Sephadex LH-20 (Sigma) column chromatography using MeOH (100%, HPLC grade) as mobile phase. Purified compounds (1–4) were identified by LC-UV/DAD co-chromatography with reference standards. A portion of each dried isolated compound was dissolved in dimethyl sulfoxide (H_2O:DMSO, 90:10 V/V) to a final concentration of 75 mg L^{-1}. The resulting solutions were used in the antibacterial studies.

2.5. Determination of minimum inhibitory concentrations

The nine strains of *N. gonorrhoeae* used in this study belong to the bacterial collection of the National Reference Laboratory of Antimicrobial Resistances (NLR-AR) (Table 1). One of the tested bacteria (5N), was susceptible (NG^S) to the antibiotics used to treat gonorrhoea, such as penicillin, tetracycline, spectinomycin, ceftriaxone and ciprofloxacin; three of the tested strains (3N, 4N and 7N), were intermediate (NG^I) to at least one of the antibiotics used; three other tested strains (1N, 2N and 8N), were characterised by plasmid-mediated resistance to penicillin due to penicillinase-production (PPNG) and the other two strains (6N and 9N), showed plasmid-mediated resistance to penicillin and tetracycline (PPNG/TRNG); 7N, 8N and 9N are reference strains, included for each of these non-susceptible phenotypes. In African countries most of the *N.gonorrhoea* strains are highly resistant to penincilin and tetraciclin but less resistant to ciprofloxacin, ceftriaxone and spectinomycin (Lewis, 2011); subsequently, the PPNG/TRNG strains were chosen according to these criteria.

Freeze-dried cell cultures were revived by culture on chocolate agar (Oxoid, Basingstoke, UK) and incubated at 35 °C in a 5% CO_2 enriched atmosphere for 24–48 hours.

The MIC for penicillin (Wyeth Lederle Portugal Farma Lda, Algés, Portugal), tetracycline, ciprofloxacin, ceftriaxone, spectinomycin (Laboratórios Atral S.A., Castanheira do Ribatejo, Portugal), as well as the extract, fractions and pure compounds, were determined by the agar dilution method, according to CLSI proceedings (Cockerill, 2011). Two controls were included for each sample: one plate in the absence of the extract solution and other in the presence of H_2O:DMSO (90:10 V/V). All experiments were carried out in triplicate to obtain consistent values. The MIC was determined as being the lowest concentration of sample that resulted in complete inhibition of growth (a slight haze of apparent growth was ignored). According to literature the MIC value for a PPNG strain is ≥2,0 mg L^{-1} of penicilin and for TRNG is ≥16,0 mg L^{-1} of tetracyclin (Martin, 2012).

3. Results

The antimicrobial activity of *S. podocarpa* root hydro-ethanol extract (Spr) and of its liquid–liquid partition fractions – diethyl ether fraction (Spr-1), ethyl acetate fraction (Spr-2) and water fraction (Spr-3), was tested against nine *N. gonorrhoeae* strains (reference and clinical isolates), expressing different susceptibilities to the common used therapeutic antibiotics (Table 1).

MIC, minimum inhibitory concentration, according to the CLSI guidelines (Paris and Nothis, 1970); Pen, penicillin; Tet, tetracycline; Cip, ciprofloxacin; Cef, ceftriaxone; Spe, spectinomycin; INSA, Instituto Nacional de Saúde; ATCC, American Type Culture Collection; CRA, Centro de Resistência aos Antibióticos; PPNG, *N. gonorrhoeae* with plasmid-mediated resistance to penicillin by penicillinase-production; NG^I, *N. gonorrhoeae* with an intermedi-