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Phytochemical and *in vitro* antimicrobial evaluation of the stem bark of *Schumanniohyton magnificum* (Rubiaceae).

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Abstract

The methanolic extract of the stem bark of *Schumanniohyton magnificum* (Rubiaceae) was screened for its phytochemical constituents and *in vitro* antimicrobial property using standard procedures. The results revealed the presence of alkaloids, flavonoids, polyphenols, sugars and steroids. A chromatographic separation of the extract led to the isolation of three compounds among which two were identified respectively to noreugenin and β -sitosterol. The extract and the identified compounds were evaluated for their antimicrobial activities *in vitro*, against some pathogenic clinical isolates, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Shigella flexneri*, *Salmonella typhimurium* and *Candida albicans*, through the disc-diffusion method. Noreugenin showed antibacterial activity against *S. typhimurium*, with an inhibition diameter of 11 mm.

Keywords: *Schumanniohyton magnificum*; medicinal plant; phytochemistry; antimicrobial activity.

1. Introduction

In their search for well-being, people transform the nature, in order to find things necessary for their survival. In this continuous process, the vegetable kingdom constitutes one among the most important source of useful substances. In fact, plants, all along their evolution, produce a great variety of chemical substances, among which some confer to species, that contain them, medicinal properties.

The use of medicinal plants by humans is very ancient, and WHO estimates that about 80% of the world population needs herbal medicines to cure diseases, especially for millions of people in the vast rural areas of developing countries [1]. In industrialized countries, 25% of medical prescriptions is made of natural origin drugs. More than 50% of free sale medicines contain medicinal plants [2-3]. These plants are used in diverse forms, including infusion, essential oils or extracts. Some famous plant derived active molecules used to date in conventional medicine include, among others, aspirin from salicin, isolated from leaves and bark of willow, quinine from the bark of *Cinchona sp.*, artemisinin from *Artemisia annua*.

In order to contribute to the discovery of active molecules of plant origin, we initiated the phytochemical study and the antimicrobial evaluation of extractives from *S. magnificum*, a species of the Rubiaceae family.

S. magnificum is widely used in African ethnomedicine in the treatment of various diseases, in particular, fever and malaria. A decoction of the stem bark is used in Cameroon in the treatment of dysentery [4], and as a lotion after circumcision [5]. In Congo, the stem bark is used as antiseptic for the treatment of blennorrhoea, syphilitic cankers or ulcers. The bark infusion is used as a purgative or vermifuge in order to relieve stomach ache [6]. The juice is used as a snake bite remedy in Nigeria, and the protective effects of the extract against snake venom have been demonstrated [7-8]. This plant is also well-known as snake repellent [9]. *S. magnificum* extracts are active against *Plasmodium falciparum* [10].

The aim of this study was to extract, isolate, purify and characterize the different chemical constituents of the stem bark of *S. magnificum*, and subsequently evaluate the antimicrobial activities of the raw extract and isolated molecules.

Many phytochemical work have been held on *S. magnificum* that resulted in the isolation and characterization of many biomolecules [4, 7-9].

Our study is justified by the fact that it focuses essentially on the antimicrobial activities of

extracts and isolated biomolecules from *S. magnificum* against clinical isolates pathogenic strains.

2. Material and methods

2.1. Plant material

Stem bark of *S. magnificum* was collected in May 2012 in the Mondah forest near Libreville, in the Estuaire Region (Gabon). The plant was identified, and a Voucher specimen (N° 2017, Mondah) was deposited in the National Herbarium of Gabon, Libreville.

2.2. Extraction

The stem barks of *S. magnificum* were dried and ground. The powder obtained (1.64 kg) was extracted at room temperature with methanol (1.5 L, 2 x 72 hours). The extract obtained was evaporated, resulting in 25.76 g of methanol extract that was thereafter submitted to phytochemical screening and chromatographic separation on a silica gel column.

2.3. Chemical screening

The plant extract was screened for its qualitative chemical composition, using standard methods described in the literature [11-12]. The identification of the following groups was considered: alkaloids, flavonoids, phenols, reducing sugars, saponosides, sterols-triterpenes and sugars.

Alkaloids 0.5 g of each extract was agitated with 5 ml of hydrochloric acid in a steam bath, then 1 ml aliquots of filtrate were treated with a few drops of Mayer's reagent or Dragendorff's reagent. The presence of a precipitate after treatment with either reagent is a preliminary indicator of the presence of alkaloids. To remove non-alkaloid compounds that could lead to false-positive reactions, part of the extract was alkalized with 40% ammonia solution then treated twice with chloroform. The second chloroform extract was concentrated and then retested with the Mayer and Dragendorff reagents. **Flavonoids** were detected by using the Shibata reaction or cyanide test. Briefly, 3 ml of extract was evaporated and the residue was dissolved in 2 ml of 50% methanol, then a few magnesium shavings and a few drops of concentrated hydrochloric acid were added. The development of a red-orange or purplish color indicates the presence of flavones aglycones. **Polyphenols** Boiled aqueous extract (1 ml) was mixed with 1% ferric chloride. A black-blue color indicates the presence of gallic tannins and a dark green color tannin catechists. When both were detected in the same extract, they were separated with Styasny's reagent. A drop of the extract was placed on a slab of silica gel and eluted in an atmosphere saturated with chloroform/acetic acid/formic acid (5:4:1). Then the plates were sprayed with 10 ml of methanol solution at 5% nitrous acid and heated in an oven at 80 °C for 10 min. The presence of tannins is revealed by the appearance of blue spots, while polyphenols are revealed by a violet-blue, pink-orange, pink-violet, or red coloration. **Reducing sugars** One milliliter of extract was dissolved in 2 ml of distilled water and 1 ml of Fehling liquor and boiled for 30 min. The formation of a brick-red precipitate indicates

the presence of reducing sugars. **Saponosides** 1% of each sample decoction was returned gradually in 10 ml test tube for a final volume of 10 ml. After two vigorous shakes, the tubes were left to stand for 15 min and the height of the foam was measured. The tube in which the height of the foam was at least 1 cm, showed the presence of saponosides. However, the height of the foam indicated the value of the foam index. **Sterols and triterpenes** these families of compounds were identified by using the Lieberman-Burchard reaction. Briefly, 0.5 g of extract was dissolved in 0.5 ml of chloroform in 0.5 ml of acetic anhydride, and cooled on ice before carefully adding sulfuric acid. A change in color from purple to blue indicates the presence of sterols, while a green or purple-red color indicates the presence of triterpenes. **Sugars:** a little quantity of the extract was dissolved in an ethanol/ α -naphthol (99%/1%) solution contained in a test tube, then allowed to run on the tube wall few drops of concentrated sulphuric acid. Sugars presence was detected by the emergence of a red ring at the interface.

For all the families tested, according to the precipitation or color intensity of each tube, following evaluations were given: (+++); (++); (+).

2.4. Fractionation and purification

20.0 g of the methanol extract obtained (25.76 g, 1.57%) was submitted to flash chromatography in a 70-230 mesh silica gel column (270 g) with stepwise gradient elution by n-hexane/EtOAc mixtures (100:0; 80:20; 50:50; 0:100), and EtOAc/MeOH mixtures (50:50; 0:100). Thirty eight column fractions of 150 ml each were collected and combined according to their TLC profiles on precoated Kiesegel 60 F₂₅₄ plates developed with CH₂Cl₂/MeOH mixtures. Four groups of fractions E1 (1-16), E2 (17-27), E3 (28-32) and E4 (33-38), respectively, were eluted. The fraction E1 (5.8 g) was dissolved in a minimum of methylene chloride and fixed on silica. After complete evaporation of the solvent, the powder obtained was separated through column chromatography over Si gel (70-230 mesh) eluting with n-hexane/EtOAc gradient of increasing polarity resulting in the constitution of four sub-fractions: F1 (2.0 g), F2 (0.3 mg), F3 (1.5 g) and F4 (0.2 g). by direct filtration, sub-fractions F1 and F3 afforded two pure compounds indexed H1 (7.6 mg) and H2 (2.5 mg) respectively.

Fraction E2 (8.35 g) after concentration and chromatography over Si gel eluting with ethyl acetate/propan-2-ol et propan-2-ol/MeOH gradients of increasing polarities afforded H1 (8 mg), H2 (9.91 mg) and H3 (1.60 mg). Fractions F3 (1.8 g) and F4 (2.4 g) according to their little quantities and the complexity and the complexity of their chemical constitutions were not submitted to any chromatographic separation.

Compounds H1 and H2, after spectroscopic analysis and comparison of their spectroscopic and physical data with those reported in the literature [9, 13], were identified respectively to β -sitosterol and noreugenin (Fig. 1). The extraction scheme is shown below (Fig. 2)

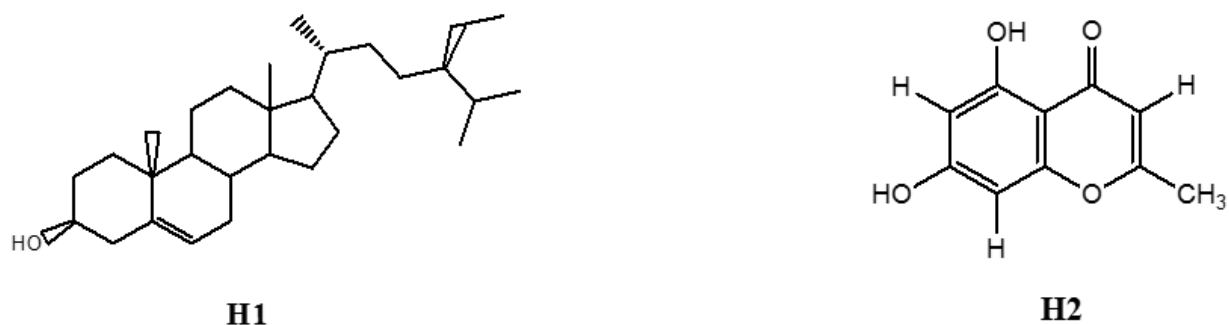


Fig 1: Chemical structures of isolated and identified compounds.

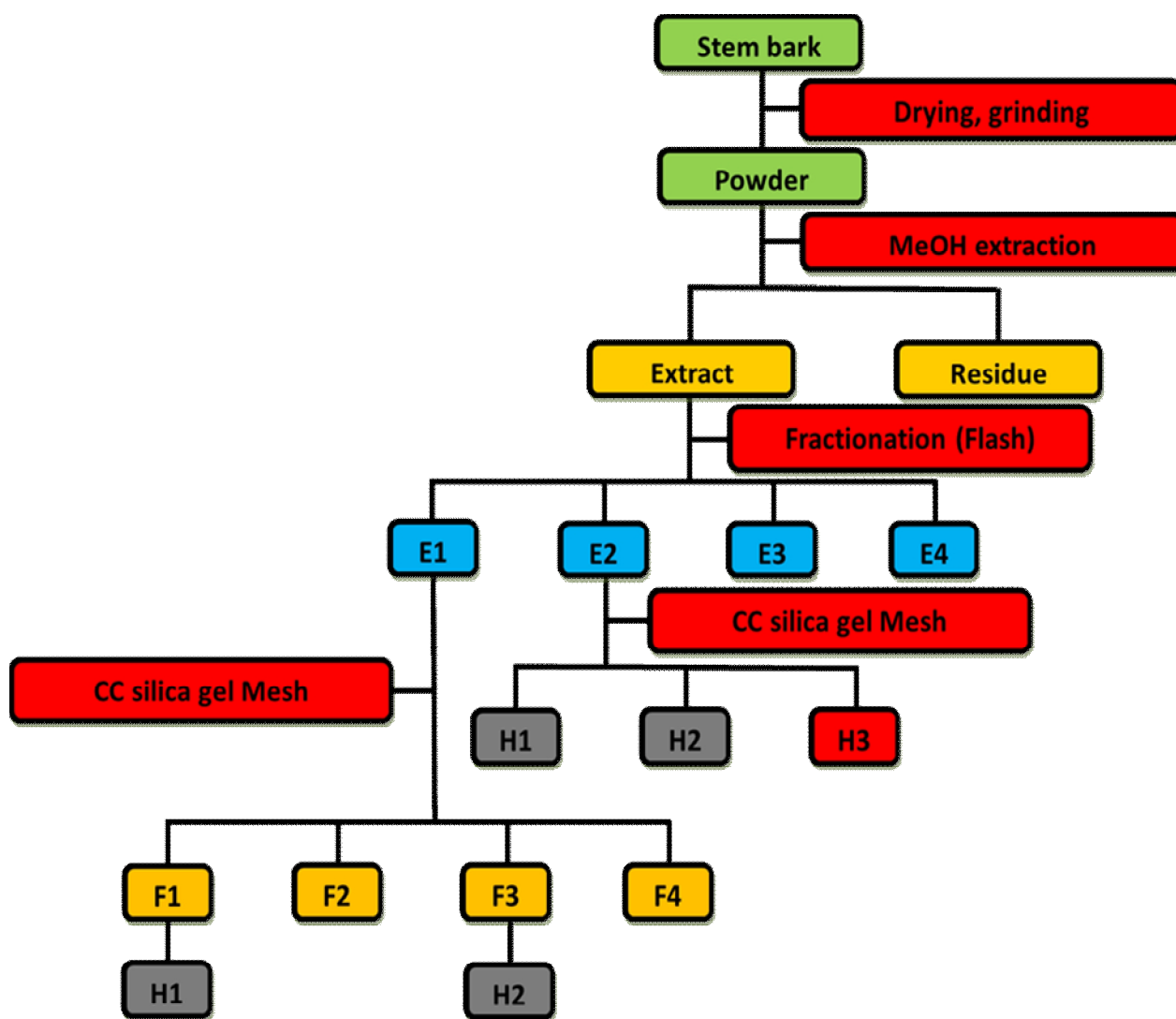


Fig 2: Extraction and fractionation scheme of the stem bark of *S. magnificum*.

2.5. Microbiological surveys

2.5.1. Microorganisms

Seven bacterial species, Gram-positive (*Staphylococcus aureus* and *Staphylococcus Saprophyticus*) and Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*,

Pseudomonas aeruginosa, *Salmonella typhimurium* and *Shigella flexneri*), and the yeast *Candida albicans* obtained from “Centre Pasteur du Cameroun”, were used for the antimicrobial tests. The strains were maintained on agar slant at 4 °C in the laboratory, where the antimicrobial tests were

performed. The strains were activated at 37 °C for 24 h on Tryptica soy agar or Sabouraud glucose agar supplemented with chloramphenicol respectively for bacteria and fungi prior to any screening. Mueller Hinton agar and Brain Heart Infusion (BHI) were used for the antimicrobial assays [14].

2.5.2. Antimicrobial activity

The antimicrobial activities of the extracts and isolated compounds were determined using the Berghe and Vlietnick agar-well diffusion method [15]. Plates containing 30 ml of sterile nutrient broth were inoculated with standardized inoculate prepared using a cell suspension of about 1.5×10^6 CFU/ml "colony forming units per milliliter". Four wells of 5 mm diameter were made on each plate with sterile Pasteur pipette and 50 μ l of the plant extract and isolated molecule at a concentration of 20 mg/ml was dispensed into each well. The sample was allowed to diffuse into the medium for 1 hour at room temperature and then, it was incubated for 24 hours at 37 °C. The zones of growth inhibition were measured and recorded in millimeter. The negative control was set up in a similar manner except that the extract was replaced with sterile distilled water. All the testing was done in duplicate. For the positive control, two reference antibiotics (amoxicillin and gentamycin) were used in a similar manner and at the same concentration (20 mg/ml).

3. Results

3.1. Qualitative analysis of phytochemical screening

Phytochemical screening using qualitative analysis on the stem bark of *S. magnificum* methanol extract showed the presence of the following constituents: alkaloids, flavonoids, polyphenols, sterols and sugars. However, reducing sugars, saponosides and triterpenes were not detected in the plant. The details on phytochemical screening results are shown in Table 1.

Table 1: Qualitative analysis of phytochemical constituents of the stem bark of *S. magnificum*.

Phytochemical constituents	Stem bark extract
Alkaloids	++
Flavonoids	++
Phenols	+++
Reducing sugars	-
Saponosides	-
Sterols	++
Sugars	++
Triterpenes	-

+++ Very intense, ++ intense, + weak, - absent.

3.2. Chromatographic and antimicrobial analysis

Chromatographic separation of the stem bark of *S. magnificum* (Rubiaceae) led to the isolation of three compounds, among which two were identified to known β -sitosterol (**H1**) and noreugenin (**H2**). The antimicrobial activities of the extract and isolated compounds have been evaluated against some pathogenic strains using the agar-well diffusion method. Inhibition diameters obtained are shown in Table 2.

S. magnificum significantly inhibited the growth of *S. thyphimurium* (ID= 12 mm); while it has no effect on *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *S. aureus*, *S. saprophyticus*, *S. flexneri* and *C. albicans*. Among different microorganisms, purified compounds β -sitosterol (**H1**) and in some extent noreugenin (**H2**) selectively inhibited the growth of *P. aeruginosa*, *S. aureus*, *S. saprophyticus* and *S. thyphimurium*. This suggest that the antimicrobial spectrum of *S. magnificum* is lesser than that of the purified compounds, noreugenin and β -sitosterol.

Table 2: Inhibition diameters of the extract and isolated compounds.

Microorganisms	Inhibition diameters (mm)				
	Extract	H1	H2	Amoxicillin	Gentamicin
<i>E. coli</i>	-	-	-	8	17
<i>P. aeruginosa</i>	-	8	8	-	26
<i>K. pneumoniae</i>	-	-	-	-	-
<i>S. aureus</i>	-	8	-	8	26
<i>S. saprophyticus</i>	-	8	-	-	-
<i>S. flexneri</i>	-	-	-	14	20
<i>S. thyphimurium</i>	12	-	11	19	26
<i>C. albicans</i>	-	-	-	-	-

4. Discussion

The chemical screening that we did, revealed that *S. magnificum* contained biomolecules potentially active against some bacterial and fungal pathogenic strains. These included steroids, which play an important function in healing scar and wound, and that have been shown to be cardiotoxic and antiparasitic [16, 17]; polyphenols, that are antibacterials, antifungals and antiseptic; alkaloids which are stimulants, antibiotics, antifungals and pest-destroying [16]. Sugars have antitussive, expectorant, analgesic, immunomodulatory and cytoprotective properties [16].

So from this observation, it can be possible to justify the common usage of *S. magnificum* in traditional medicine for the treatment of several diseases by the presence in its stem bark of many potentially active biomolecules, including steroids, phenols, flavonoids and alkaloids. The confirmation of this came from the results obtained after *in vitro* antimicrobial analysis of the extract and isolated molecules. Thus, the crude extract has a good inhibitory activity on the growth of *S. thyphimurium* with an inhibition diameters of 12 mm. In the same way, compound **H2** presented inhibitory activity on the growth of the same strain with an inhibition

diameter of 11 mm. *P. aeruginosa*, *S. aureus* and *S. saprophyticus* presented a weak sensitivity vis-à-vis compound **H1**, as well as *P. aeruginosa* for **H1**. Overall, *C. albicans*, and *K. pneumoniae* appeared to be insensitive to **H1**, **H2**, amoxicillin and gentamycin. However, the diameters of inhibition induced by **H1**, **H2** and amoxicillin remain lower than those of gentamycin for all strains.

The almost identical activities of the crude extract and **H2** on *S. typhimurium*, suggests that **H2** would be one of the principal antibacterial active principles of *S. magnificum*.

These results partially validate the usage of this plant as antiseptic in traditional medicine.

Besides, it has been shown that *S. magnificum* possessed antioxidant activity [18]. This property can be justify by the presence in its stem bark of phenols, especially flavonoids that have been reported as protective antioxidants at various levels and potential membrane lipids protectors from oxidation [17]. It could be inferred that *S. magnificum* as some vegetal species, possesses the potential to be used to treat or prevent degenerative diseases where oxidative stress is implicated.

5. Conclusion

The phytochemical study of the stem bark of *S. magnificum*, allowed us to isolate three compounds, among which, two were identified to β -sitosterol and noreugenin. Antimicrobial evaluation of the crude extract and compounds showed antibacterial activity of noreugenin against *S. typhimurium*, and the crude extract against the same strain, suggesting that noreugenin could be an antibacterial active principle of *S. magnificum*. The antimicrobial activities of the crude extracts and isolated biomolecules of this plant, partially validate the use of *S. magnificum* in traditional medicine. However, more biological studies, including the determination of minimum bactericidal or fungicidal concentrations are necessary to evaluate the real effects of the studied samples on the growth of different microorganisms, and therefore the real contribution of *S. magnificum* in the treatment of various infectious diseases.

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