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PHYTOCHEMICAL SCREENING, ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF AQUEOUS AND ORGANICS STEM EXTRACTS OF *STROPHANTHUS HISPIDUS* DC.

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ABSTRACT: Antibacterial and antioxidant activities of *Strophanthus hispidus* stem aqueous and organic (cyclohexane, chloroform, ethyl acetate and ethanol) extracts were investigated in order to assess its use in traditional medicine. *In vitro* antibacterial activity assessed by Disc diffusion and Microdilution methods, was tested against Gram positive bacteria (sensible *Staphylococcus aureus*) and gram negative bacteria (*Escherichia coli* and *Pseudomas aeruginosa*) at a concentration of 0.5 mg/ml. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) values for all extracts range respectively from 0.05 mg/mL to 7.5 mg/mL and 0.117 mg/mL to 7.5 mg/mL. The activity was higher for cyclohexane and aqueous extracts. Cyclohexane extract had only terpenoids in considerable amount whereas aqueous extract contain alkaloids anthraquinone, tannin, saponine and polyphenols. Antioxidant activity evaluated using 2,2-diphenyl-2-picryl- hydrazyle (DPPH) revealed that only ethanolic extract had appreciable antioxidant activity with an IC_{50} value of 37,9 µg/mL. Ethanolic extract contain polyphenol, flavonoids, tannins, alkaloids, terpenoids and saponines. The presence of these secondary metabolites explains the use of this plant in traditional medicine.

INTRODUCTION: Medicinal plants are best source of new pharmaceuticals and health care products. According to World Health Organization (WHO), herbal drugs are being used by 75-80% of World population, especially in developing countries [1], hence the interest of screening medicinal plants for bioactive compounds.

Nowadays resistance of pathogens against antibiotics and oxidative stress caused by free radicals, have raised a great interest in the search of new antibacterial and antioxidant compounds from nature [2, 3]. Natural crude drug extracts isolated from plant species can be prolific sources for such new drugs.

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The *Strophanthus hispidus* which belongs to the Apocynaceae family is found all over Africa (D.R. Congo, Senegal, Ghana, Sudan, Uganda and Tanzania) in savannah and forests. The roots, stem barks and leaves of *S. hispidus* are traditionally used in the treatment of Syphilis ulcers, bony syphilis, guinea-worm sores, wounds, arthritis, stroke, heart failure, rheumatism, antidote to snake-venom and skin diseases [4, 5]**.** The roots and leaves methanolic extracts have been found to have antimicrobial and antioxidant activities *in vitro* [6, 7]. However, the stem bark has received less attention to our knowledge therefore this study aim to investigate antioxidant and antibacterial activities of *S.hispidus* stem barks aqueous and organic extracts.

MATERIAL AND METHODS:

Plant Materials: The stem barks of *S. hispidus*were collected in April 2014 from their natural habitats in Mayala village Kongo Central (DRC).

The collected plant materials were authenticated by INERA (Institute National d' Etudeset Recherches Agronomiques) Herbarium at Faculty of Science, University of Kinshasa. The stems bark were dried at room temperature for two weeks then milled into powdered used to prepare extracts.

Preparation of extracts:

Aqueous extract: Seven grams (7 g) of powdered material were soaked in 105 mL of water for 2h at 40°C. The mixture was then filtered with Whatmann N°1 paper. The filtrate was concentrated under vacuum to yield 0.729 g (10,29 %) of paste material.

Organic extracts: Fifteen grams (15g) of powdered materials were successively macerated using four solvents (150mL for each solvent) in ascending order of their polarity (cyclohexane, chloroform, ethyl acetate and ethanol). The fractions were filtered twice using Whatman N° 1 paper and the filtrate was evaporated under reduced pressure (using a Rotary Evaporator). The yields of the cyclohexane, chloroform, ethyl acetate and ethanol stem extract of *S.hispidus* were respectively 4.06, 3.07, 2, and 2.73 % w/w (related to the dried material). All extracts were stored in refrigerator before used.

Phytochemical screening of the plant extracts: One hundred milligrammes (100 mg) of each dry extract was used for screening following the bioactives compound: tannins, flavonoids, alkaloids, polyphenols, saponins, terpenoids, anthraquinones, and steroids according to the method described by Ayoola [5].

Determination of antibacterial activity: Standard bacterial cultures like *Staphylococcus aureus* (ATCC 25923, gram positive), *Escherichia coli* (ATCC 25922, gram negative), and *Pseudomonas aeruginosa* (ATCC 27853, gram negative) were obtained from INRB (Institute National de Recherche Biomedicale) Kinshasa. The bacterial stock cultures were maintained on Muller Hinton Agar, which were stocked at 4°C. Three to five similar colonies were selected from the stock and transferred using loop into 8 mL of sterile TSB (Trypton Soja Broth) and incubated for 24 hours at 37°C. The antibacterial assays were carried out by the disc-diffusion and micro dilution methods.

Disc diffusion method: Antibacterial screening is generally performed by disc diffusion method [8], which is a qualitative test. Twenty (20) mL of Mueller Hinton agar were plated in petri dish with 100 µL of each bacterial culture. Sterile Filter paper discs (6 mm in diameter) impregnated with 20 µL of plant extracts (500µg/mL) were placed on test organism-seeded plates. Tween-80 (distilled water was added to facilitate dissolution) was used to dissolve the extract. Blank disc impregnated with solvent (Tween-80) was used as negative standard. The activity was determined after 18h of incubation at 37°C. The diameters of inhibition zone produced by the extract were measured with a ruler. Clear inhibition zones around the discs indicated the presence of antimicrobial activity. Ciprofloxacin 10 µg/mL was used as positive standard. Each sample was used in triplicate.

Microdilution Method: The MICs (concentration which completely inhibit bacterial) of the *S. hispidus* stem barks extracts against the test bacteria were determined using the modified microdilution technique as described by Agyare et al.[7]

Under aseptic conditions, 96 wells microplates were used. All the wells of microplate were filled with 50µl of nutrient broth (Trypton Soja Broth).Test solutions (15mg/mL) of the extracts were prepared with Tween 80-Steriled water and 50 μL of this test solution were serially diluted to29 μg/mL in the microplate's wells. Finally, 10μL (10^6cfu/mL) of the inoculums were added to each well of the microplates. The covered microplates were incubated at 37°C for 24h. To indicate growth, 5μL of resazurin dissolved in water was added to the microplate's wells and incubated at 37°C for 30min. All experiments were performed in triplicates.

The minimum bactericidal concentrations (MBCs) were determined by subcultivation. 10 µL of well's contents were placed in petri dish which restrained 100 µL of Typic Soja Agar (TSA) and incubated for 18-24h at 37°C. The lowest concentration with no visible growth was defined as MBC, indicating = 99.9% killing of the original inoculum.

Determination of antioxidant activity: *S.hispidus* stems extracts stock solution was prepared in

ethanol at a concentration of 1000µg/mL (1mg/mL). From the stock solution various concentrations 20, 30, 40, 50, 60, 70, 80 and 90 µg/mL were obtained. Free radical scavenging activity of stem extract was measured using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) according to modified method of Fatema [9]. The DPPH solution (30 mg/mL) was prepared in ethanol and 1 mL of this solution was added to 9 mL of various concentrations stem extracts and ascorbic acid as reference compound at 0.001, 0.002, 0.003, 0.004, 0.005, 0.006 and 0.007 mg/mL. After 30 min in the dark, absorbance was measured at 517 nm by UV spectrophotometer. An equal amount of DPPH and Ethanol served as blank solution control. All the tests were performed in triplicate and the graph was plotted with the mean value. The percentage of inhibition was calculated by comparing the absorbance values of control blank solution to that of samples. The percentage scavenging activity was calculated using the following formula:

$$\text{Inhibition (\%)} = [(A_o - A_s)/A_o] \times 100$$

Where A_o is the absorption of the blank and A_s the absorption of extract.

Inhibitory Concentration: IC_{50} is the amount (µg/mL) reducing the absorbance by 50 % was obtained from a plot of concentration in µg/mL to % of inhibition.

RESULTS AND DISCUSSION:

Phytochemical Screening of *S. hispidus:* Results of chemical screening of aqueous and organic stem extracts of *S. hispidus* shown in **Table 1** revealed the presence of alkaloids, flavonoids, tannins, anthraquinones, polyphenols, terpenoids and saponins. Repartition of these metabolites in different extracts depends very much on solvent polarity. The less polar cyclohexane has only terpenoids in large amount whereas the more polar aqueous and ethanol extracts had all the metabolites except flavonoids for the aqueous extract and anthraquinone for the ethanolic extract. The metabolites found in the ethanolic extract were identical to those in the methanolic extracts of leaves and roots of the same plant [7].

TABLE 1: PHYTOCHEMICAL CONSTITUENTS OF DIFFERENT STEM EXTRACTS OF *S.HISPIDUS*.

Phytochemical Tests	Aqueous	Ethanol	Ethylacetate	Chloroform	Cyclohexane
Alkaloids	+	+	-	-	-
Flavonoids	-	+	+	-	-
Tannins	+	+	+	+	-
Anthraquinones	+	-	-	-	-
Polyphenols	+	+	+	+	-
Terpenoids		+	+	+	+++
Saponins	+	+	+	-	-

+: presence of secondary metabolite; +++: abundance presence of secondary metabolites; - : absence of secondary metabolite.

Determination of antibacterial activity:

Disc diffusion method: Disc diffusion methods are extensively used to investigate the antibacterial activity of natural substances and plant extracts. These assays are based on the use of discs as reservoirs containing solutions of substances. In the case of solutions with a low activity, however, a large concentration or volume is needed. The limited capacity of discs means that holes or cylinders are preferably used. The results of antibacterial activity of the aqueous and organic stem extracts of *S. hispidus* against *E. coli*, *P. aeruginosa* and *S. aureus* are reported in **Table 2**. All bacterial species were found to be sensible to the different stem extracts at the tested concentration of 0.5mg/mL. Cyclohexane extract showed more antibacterial activity with the three bacteria with inhibitory zones of 13.25 mm, 12.50 mm and 12.55mm respectively for *E.coli*, *P. aeruginosa* and *S. aureus*. The aqueous extracts second in efficacy showed mean inhibitory zones of 12.13mm 12.12 mm and 12.30 mm respectively for *E.coli*, *P. aeruginosa* and *S. aureus*. In general the inhibitory zones observed for all the stem extracts of *S.hispidus* were in the same range of that of methanolic extracts of leaves and roots of the same plant. There was no inhibition zone for Tween80 used as negative standard, whereas ciprofloxacin used as positive standard (10µg/mL) had the widest inhibitory zones of 32.79 mm, 30.28 mm and 31.50 mm respectively for *E.coli*, *P. aeruginosa* and *S. aureus.*

TABLE 2: DIAMETER OF INHIBITION VALUES (MM) OF THE DIFFERENT STEM EXTRACTS OF *S. HISPIDUS*

Bacteria / Extracts	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Aqueous	12.13 ± 0.24	12.12 ± 0.51	12.30 ± 0.33
Ethanol	10.32 ± 0.12	11.82 ± 0.55	11.03 ± 0.39
Ethylacetate	10.36 ± 0.52	11.58 ± 0.36	12.15 ± 0.15
Chloroform	11.32 ± 0.16	11.04 ± 0.21	11.12 ± 0.35
Cyclohexane	13.25 ± 0.30	12.50 ± 0.51	12.55 ± 0.36
Ciprofloxacin	32.79 ± 0.67	30.28 ± 0.72	31.50 ± 0.58
Tween80-distilled water (7:3)	00.00 ± 00	00.00 ± 00	00.00 ± 00

Microdilution method: MIC, MBC and MBC/MIC values for different extracts obtained for the pathogenic bacteria species are reported in **Table 3**. All the extracts were found to be active against the test organisms. The minimum inhibitory concentration (MIC), the lowest concentration of crude extract at which no microbial growth, values supported data obtained from disc diffusion assay *E.coli* was very sensitive to cyclohexane and aqueous extracts with a MIC values of respectively 0.058 mg/ml and 0.117 mg/mL. The values of MICs for the aqueous and cyclohexane extracts were the same for *P.aeruginosa* and *S.aureus* 0.058 mg/mL and 0.1172 mg/mL respectively.

From this study, it was found that the *S.hispidus* stem extracts exhibited strong and broad spectrum antimicrobial activity against these pathogens. Since all of the MICs of the extracts against the test organisms were below 8mg/mL, it could be inferred that the extracts exhibited potent antimicrobial activity according to Fabry and his colleagues [10].

Minimum bactericidal concentration (MBC) for each extract is the lowest dilution level of extract needed to completely inhibit bacterial growth, depend on the solvent and the bacteria. The lowest values were obtained from aqueous and cyclohexane extracts. For aqueous extract MBC values were respectively 0.234mg/mL, 0.234mg/mL and 0.468mg/mL against *E.coli*, *P.aureginosa* and *S.aureus*. For cyclohexane extract MBC values were respectively 0.117mg/mL, 0.117mg/mL and 0.234mg/mL for *E.coli, P.aureoginosa* and *S.aureus*. Results of MBC assay were similar to data obtained from disc diffusion assay and MIC determination. The fact that the ratio of MBC/MIC for all *S.Hisidus* stem extracts are below 4 is a clear indication of the large bacteridal activity of all extracts. The antimicrobial action of the extracts may be attributed to astringen nature of the phenolic constituents including tannins, anthraquinones and other secondary metabolites such as terpenoids, alkaloids present in the extracts [11, 12, 13].

TABLE 3: MIC, MBC AND MBC/MIC OF STEM EXTRACTS AGAINST THE PATHOGENIC BACTERIA BY MICRODILUTION ASSAY

Bacterial strains	Extracts	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC
Escherichia coli	Aqueous	0.1172	0.2343	1.99
	Ethanol	7.5	7.5	1
	Ethyl acetate	7.5	7.5	1
	Chloroform	3.75	7.5	2
	Cyclohexane	0.058	0.1172	2.01
Pseudomonas aeruginosa	Aqueous	0.058	0.2343	4
	Ethanol	0.4682	1.875	4
	Ethyl acetate	0.4682	0.9375	2
	Chloroform	0.9375	1.875	2
	Cyclohexane	0.058	0.1172	1
Staphylococcus aureus	Aqueous	0.1172	0.4687	3.99
	Ethanol	1.85	3.75	2
	Ethyl acetate	0.1172	0.2343	2
	Chloroform	3.75	3.75	1
	Cyclohexane	0.1172	0.2343	2

Test for antioxidant activity:

DPPH Radical Scavenging activity: The free radical scavenging activity of *S. hispidus* stem extracts was studied by its ability to reduce the DPPH, a stable free radical. DPPH is a free radical and it gives a strong absorption band at 517nm in the visible region of the electromagnetic radiation. Screening for antioxidant activity was negative for all the stem extracts except for the ethanol extract where the color of the DPPH changed from violet to yellowish. The IC_{50} value determined was 37.9µg/mL, whereas that of ascorbic acid used as reference was 4.17µg/mL.

These results suggest that ethanol extracts possess significant antioxidant properties. The IC_{50} reported here for the ethanolic extract is lower to those reported by C. Agyare for methanolic extracts of leaves (49.8µg/mL) and roots (45.1µg/mL) of the same plants.[7]

Antioxidant activity of *S.hispidus* ethanol stem extract may be attributed to flavonoids and polyphenols present in the extract [5]. These constituents in the extracts play a major role in preventing and protecting oxidative damage from free radicals [14, 15, 16].

CONCLUSION: All the stem extracts of *S. hispidus* exhibited antimicrobial activities with MIC ranges from 0.011 to **7**.5mg/mL and MCB value from 0.117 to 7.5mg/mL. 0.4682 to 7.5 mg/mL**.** Aqueous and cyclohexane extracts exhibited similar antibacterial activity and their MICs were in the same range.

Only ethanol stem extract exhibited free radical scavenging activity when screened with DPPH and showed free radical scavenging activity with IC_{50} value of 37.9µg/mL. These pharmacological properties may justify traditional uses of this plant for treatment of microbial infections, wounds, cardiovascular, cancer, asthma, inflammatory conditions and macular degeneration. Further work will explore bio-guided fractionation of the most antibacterial extracts (aqueous and cyclohexane) in order to isolate the biological most active compounds.

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