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HPLC Fingerprint profile and Antioxidant, Antibacterial Activities of Methanol Extract of *Strophanthus hispidus* DC (Stem bark).

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Abstract
Plant extracts with antioxidant and antibacterial activities are an important research area in the current medical world, aiming to isolate active components in order to develop new chemotherapeutic agents that can be used in the treatment of various diseases. In the present study we determined antioxidant, antibacterial activities of methanol extract of Strophanthus hispidus DC (Stem bark) and his HPLC fingerprint profile. Antioxidant activity evaluated using 2,2-diphenyl2-picryl- hydrazyle (DPPH) revealed that methanolic extract had a significant antioxidant activity with an IC50 value of 34.63 µg/mL. The in vitro antibacterial activity assessed by Microdilution methods, was tested against Gram positive bacteria (sensible Staphylococcus aureus) and gram negative bacteria (Escherichia coli and Pseudomonas aeruginosa) at different concentrations ranging from 0.029 to 3.75 mg/mL. Better antibacterial activity was observed against negative gram bacteria E.coli and P.aureginosa with the MIC values of 0.468mg/mL and 0.234 mg/mL, respectively. The MIC value of the extract against P.aureginosa bacteria strain is similar to that of the standard (Chloramphenicol) against the same bacterial strain. The total phenolic content of the methanolic extract was performed using the Folin-Ciocalteu reagent and its value was 54.91g GAE/100 g. This extract was found to contain tannins in large amount, flavonoids, alkaloids, saponins, steroids and Glycosides. HPLC finger-printing of this extract was developed and showed seven major compounds including ascorbic acid, quercetin, resorcinol and gallic acid represented in large amount. The presence of these secondary metabolites explains the use of this plant in traditional medicine and could be used in the future development as antioxidant and antibacterial agent.

Keywords: *Strophanthus hispidus DC, antioxidant, antibacterial, phytochemicals, TLC, HPLC.*

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I. 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.

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, and like antidote to snakevenom and skin diseases[4, 5]. The roots and leaves methanolic extracts have been found to have antimicrobial and antioxidant activities *in vitro*[6, 7]. The plant contains an amorphous glycoside (pseudo-strophanthin) with heavy oil, two alkaloids (trigonelline and choline), resin, mucilage, and a Rhamnose sugar[5].

The roots and leaves methanolic extracts have been found to have antimicrobial and antioxidant activities *in vitro*[6, 7]. This study aim to investigate antioxidant, antibacterial activities of *S.hispidus* stem barks methanolic extract and his HPLC fingerprint profile.

II. Material And Methods:

Plant Materials and Chemicals:

The stem barks of S. hispidus were collected in December 2018 from their natural habitats in Mayala village, Kongo Central (DRC). The collected plant materials were authenticated by INERA (Institute National d'Etudes et Recherches Agronomiques) Herbarium at Faculty of Science, University of Kinshasa. Unless stated otherwise, all the chemicals were purchased from Sigma (Deisenhofen, Germany).

Soxhlet extraction

Methanol extracts of *Strophanthus hispidus* (Stem barks) was obtained by Soxhlet extraction as previously described[8]. Extracts were concentrated on a rotary evaporator and the resulting residue stored at -20 ˚C until needed. Stock solutions of 20 mg/mL of *Strophanthus hispidus* (Stem barks) extract was prepared in 99% methanol and diluted as needed for different assays. Diluted extract solution was filtered and sterilized before use.

Thin-layer chromatography (TLC)

Silica gel thin-layer chromatography (TLC) was employed to estimate the approximate number of distinct chemical entities within each extract. Briefly, a small sample of the stock extract solutions were dissolved in 1 mL methanol and spotted on an in-house prepared 10 cm by 5 cm and 0.2 mm thick silica gel plate as previously described[8]. Developing solvent system utilized for the separation of constituents was ethyl acetate and petroleum ether in a 4:1 ratio. Iodine vapor visualization of resolved chromatographic bands and calculation of Rf values of constituent bands were performed as described in the literature[8].

HPLC Finger-Printing of Extracts

The HPLC finger-printing of the methanol extracts was performed on Shimadzu LC-10 AT VP, Luna 5u C18 reverse-phase analytical column (250×4.6 mm) with binary gradient mode, SPD-M10A VP photo diode array detector (PDA), injection volume 20 μL, total flow 1 mL/min, column oven temperature 25°C and detection wavelength 280 nm. Fifty five milligrams per milliliter (55mg/mL) of extract were dissolved in 3 ml of methanol for the analysis. Ascorbic acid, gallic acid, resorcinol and quercetin were used as standard. Eluent A (acetonitrile); eluent B (0.1% phosphoric acid in water); Gradient elution program was begun with 92% of solvent B and was held at this concentration for 0–25 min. This was followed by 78% of solvent B for the next 25–40 min. Total run time was 40 min. Gradient elution of standards: 92% of solvent B (0-5 min) and 78% of solvent B (5-20 min).

Phytochemical analyses

Phytochemical composition of each crude extracts was assessed using slight modifications of the methods described by Ayoola[5].

Total phenolic content by Folin-Ciocalteu method

Estimation of the total phenolic contents of the methanolic extract was performed using the Folin-Ciocalteu reagent with protocols published elsewhere[8]. Interpolation from the standard Gallic Acid curve provided total phenolic contents of samples in Gallic Acid equivalents (GAE). A triplicate test for each sample concentration presents each sample point as a Mean ± SD.

DPPH radical scavenging assay

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 of 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: Inhibition (%) = [(Ao - As)/Ao] x 100. Where Ao is the absorption of the blank and As the absorption of extract.

Inhibitory Concentration:
IC50, the amount (µg/mL) reducing the absorbance by 50 % was obtained from a plot of concentration in µg/mL to % of inhibition.

Determination of antibacterial activity:

Standard bacterial cultures of *Staphylococcus aureus* (ATCC 25923, gram positive), *Escherichia coli* (ATCC 25922, gram negative), and *Pseudomonas aeruginosa* (ATCC 27853, gram negative) were used. 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 micro dilution method.

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 (3.75mg/mL) of the extracts were prepared with Tween 80-Steriled water and 50 μL of this test solution were serially diluted to 0.029 mg/mL in the microplate's wells. Finally, 10μL (106 cfu/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. Ten microliter (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.

III. Results And Discussion:

Thin-layer chromatography (TLC) and Preliminary Phytochemical Screening

TLC profiling of the extracts gives an impressive result that directing towards the presence of number of phytochemicals. Various phytochemicals gives different Rf values in different solvent system. This variation in Rf values of the phytochemicals provides a very important clue in understanding their polarity and also helps in the selection of appropriate solvent system for separation of compounds by preparative-TLC. Compound showing high Rf value in less polar solvent system have low polarity and with less Rf value have high polarity. Mixture of solvents with variable polarity in different ratio can be used for separation of pure compound from plant extract. The selection of appropriate solvent system for a particular plant extracts can only be achieved by analyzing the Rf values of compounds in different solvent system.

Thin Layer Chromatography demonstrated that each plant extract comprise multiple chemical entities and is in line with the observed varied phytochemical compositions of the extract. The six observable TLC bands (spots) for *Strophanthus hispidus* (Stem bark) extract (Table 1) are on the lower scale of chemical entities present in the extract and the problem might be due to the lower separator efficiencies of the utilized chromatographic method. Nevertheless, Rf values in table 1 show that all chromatographic bands were reasonably well-resolved. These spots (6) indicated the presence of six major groups of phytochemical constituents in this methanolic extract.

Additional confirmatory evidence supportive of the TLC is provided by the phytochemical composition that denoted a multiplicity of functional groups embedded, potentially, in different chemical entities.

Results of chemical screening of methanolic extract of *S. hispidus* (stem bark) shown in Table 1 revealed the presence of tannins in large amount, alkaloids, flavonoids, saponins, steroids and Glycosides. The metabolites found in the methanolic extract of stem bark were identical to those in the methanolic and ethanolic extracts of leaves and roots of the same plant[5,7,10]. These phytochemical molecules are potentially bioactive.

Table 1: Thin layer chromatography (TLC) and phytochemical contents report of methanolic extracts of *Strophanthus hispidus* (Stem barks).

Sample	TLC Result Number of spots from TLC and Rf values.	Phytochemicals screening	
Methanolic extract of *Strophantus hispidus* (Stem barks).	Six(6) 0.07, 0.18, 0.34, 0.46, 0.57, 0.69	Alkaloids	+
		Flavonoids	+
		Tannins	+++

		Anthraquinones	-
		Steroids	+
		Glycosides	+
		Saponins	+

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

Total phenolic content by Folin-Ciocalteu method

The total phenolic content in methanolic extract of *S.hispidus* (stem barks) was evaluated quantitatively according to the standard *in vitro* spectrophotometrical-based Folin-Ciocalteu colorimetric assay as described in Materials and Methods section. Interpolation from the standard Gallic Acid curve provided total phenolic contents of samples in Gallic Acid equivalents (GAE).

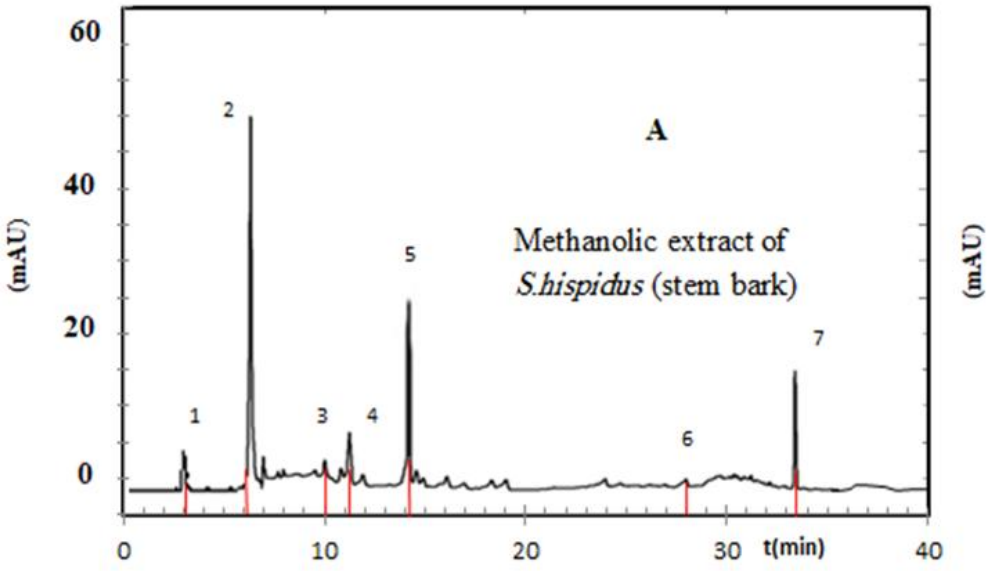
The data shows that methanolic extract of *S.hispidus* (stem bark) is a polyphenol-rich plant whose total phenols exist in structurally distinct forms as Tannins and Flavonoids. Total phenolic content of methanolic extracts was 54.91g GAE/100 g.

HPLC Finger-Printing of methanolic extract of *Strophanthus hispidus* (Stem barks).

The HPLC finger printing of the extract was determined to identify the major peaks (compounds) in the extract for the purposes of identification and quality control (Figure 1). HPLC profiles of methanolic extract of *S.hispidus* (stem bark) was analyzed for four phenolic compounds viz., Ascorbic acid, Gallic acid, Resorcinol and Quercetin.

All of these four phenolic compounds were present in the methanolic extract of *S.hispidus* (stem bark) with different retention times (RT) of 2.98 (Peak 1), 6.17 (Peak2), 10.95 (Peak 4), and 13.96(Peak 5) for Ascorbic acid, Gallic acid, Resorcinol and Quercetin respectively.

The HPLC chromatogram showed seven major peaks at the retention times (min.) of 3.11, 6.23, 10.44, 11.21, 14.12, 28.36, and 33.58 respectively at wavelength of 280 nm (Figure 1) and the major peak with a retention time (min.) of 6.2. The retention time of this peak corresponds to that of Gallic acid (6.1 minutes) used as a standard in the same solvent system. These results are similar to the phytochemical screening which showed the presence of Tannins in the large amount.



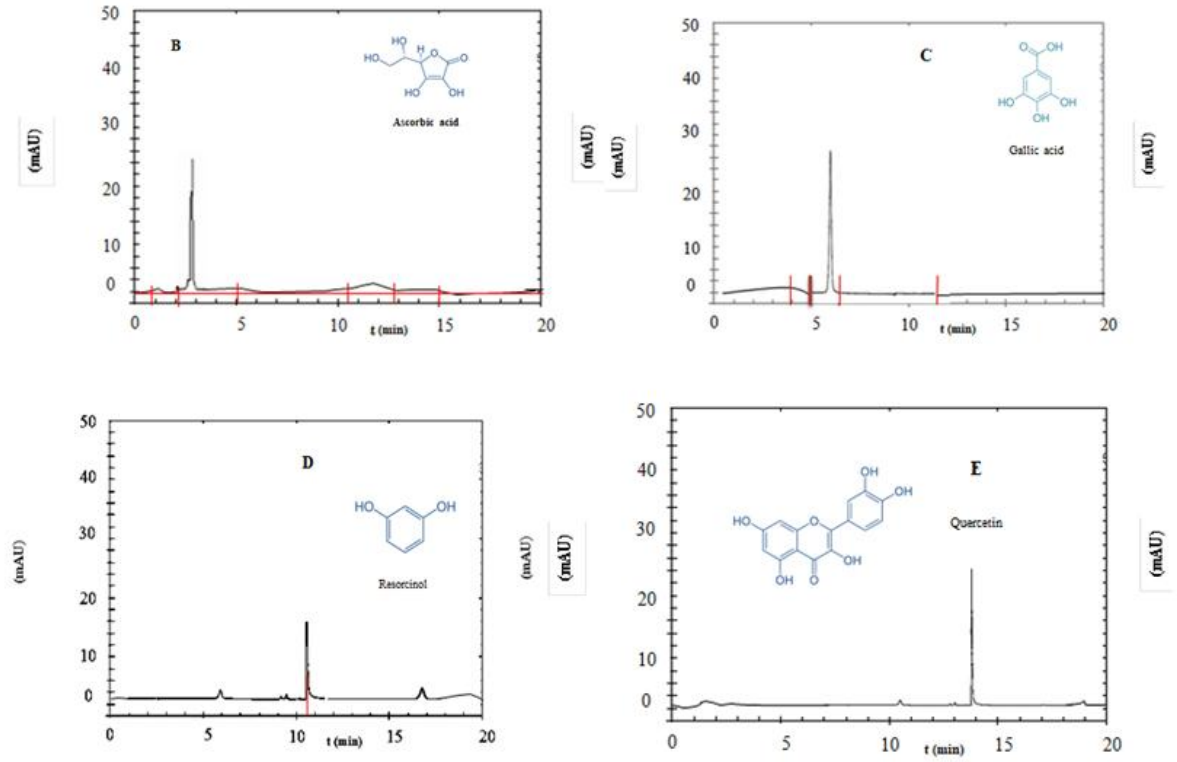


Figure 1: HPLC chromatogram (finger-printing) (A) Methanol stem bark extract of *S. hispidus*, (B) Ascorbic acid, (C) Gallic acid; (D) Resorcinol; (E) Quercetine at λ 280 nm.

Antibacterial activity

MIC, MBC and MBC/MIC values of methanol stem bark extract of *S. hispidus* against the pathogenic bacteria species are reported in Table 2. The methanol extract was found to be active against the test organisms. The minimum inhibitory concentration (MIC) is the lowest concentration of the extract at which no microbial survive.

From this study, it was found that the methanol stem bark extract of *S. hispidus* exhibited strong and broad spectrum antibacterial activity against negative gram bacteria *E.coli* and *P.aureginosa* with the MIC values of 0.468mg/mL and 0.234 mg/mL, respectively. This MIC value of the extract against *P.aureginosa* bacteria strain is similar to that of the standard (Chloramphenicol) against the same bacterial strain. The data shows no preferential display of sensitivity to the anti-proliferative effects of *Strophanthus hispidus* by the negative gram bacteria. *Staphylococcus aureus*, a positive gram bacteria strains was the least sensitive pathogenic bacteria to *Strophanthus hispidus* methanolic extract's anti-proliferative activities with a MIC value of 1.875 mg/mL. According to these results, it could be inferred that this extract exhibited potent antimicrobial activity[7,11]. The antibacterial activity of the methanolic extract against *P. aeruginosa* was better than the activity exhibited by ethanol and ethyl acetate extracts of *Strophanthus hispidus* (Stem barks) as reported by Mulula et al.[12]. This could be explained by the solubility of polyphenolic compounds in the methanol.

Minimum bactericidal concentration (MBC) of an extract is the lowest dilution level of extract needed to completely inhibit bacterial growth and depend on the solvent and the bacteria. The lowest values were obtained against negative gram bacteria *E.coli* and *P.aureginosa* with the MIB values of 0.938mg/mL and 0.234 mg/mL, respectively.

The fact that the ratios of MBC/MIC for methanol stem bark extract of *S. hispidus* is below to 4 is a clear indication of the large bactericidal activity of methanol extract. The antimicrobial action of the extracts may be attributed to astringent nature of the phenolic constituents including tannins, flavonoids, anthraquinones and other secondary metabolites such as terpenoids, alkaloids present in the extracts[11].

Table 2: MIC, MBC and MBC/MIC of stem extracts against the pathogenic bacteria by Microdilution assay.

Sample/ Standard	Bacterial strains	Concentrations (mg/mL)								MIC (mg/mL)	MBC (mg/mL)	MBC/MIC
		3.75	1.875	0.938	0.468	0.234	0.117	0.058	0.029			
Methanolic Extract *S. hispidus* Stem barks	*P. aeruginosa*	-	-	-	-	-	+	+	+	0.234	0.234	1
	E. coli	-	-	-	-	+	+	+	+	0.468	0.938	2
	S. aureus	-	-	+	+	+	+	+	+	1.875	3.75	2
Chloramphenicol	*P. aeruginosa*	-	-	-	-	-	+	+	+	0.234	0.234	1
	E. coli	-	-	-	-	-	-	-	+	0.058	0.117	2
	S. aureus	-	-	-	-	-	-	-	+	0.058	0.058	1

(+) indicates microbial growth; (-) indicates no microbial growth

Test for antioxidant activity: DPPH Radical Scavenging activity

In recent years much attention has been devoted to natural antioxidant and their association with health benefits[13,14].

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 positive for the methanolic extract of *S.hispidus* (stem bark) and the color of the DPPH changed from violet to yellowish. The IC50 value determined was 34.63µg/mL, whereas that of ascorbic acid used as reference was 3.07µg/mL. These results suggest that methanolic extract possess significant antioxidant properties. The IC50 reported here for the methanolic extract of *S.hispidus* (stem bark) is lower to those reported by C. Agyare for methanolic extracts of leaves (49.8µg/mL) and roots (45.1µg/mL) of the same plant [7]. In additional, this IC50 is also lower those reported by Mulula for ethanolic extract of stem barks (37.9µg/mL) [12].

Total phenolic contents of botanical extracts can be reliable predictors of antioxidant activities as a linear correlation of total phenolic content with antioxidant effects is a uniformly acknowledged relationship [5]. Hence, the constituents of the extracts, such as tannins and flavonoids, play a major role by preventing and protecting oxidative damage from free radicals [15, 16].

Following the quantitative determination of the total phenolic compounds in this extract with a value of 54.91g GAE/100g, as well as the HPLC analysis which showed the presence of four phenolic compounds (ascorbic acid, gallic acid, resorcinol and Quercetin), we can assume that these phenols compounds could contribute to the antioxidant properties of the methanolic extract of *S.hispidus DC* (stem bark).

IV. Conclusion

In conclusion, this study provides new scientific information about the methanolic extract of *Strophanthus hispidus DC* (stem bark), based on its antioxidant, antibacterial potentials and chemical profiling that has never been reported. The methanol stem bark of *S.hispidus DC* exhibited a high antibacterial activity against negative gram bacteria *E.coli* and *P.aureginosa* with the MIC values of 0.468mg/mL and 0.234 mg/mL, respectively. In addition, he showed also a significant antioxidant activity with an IC50 value of 34.63µg/mL.

The present study reported the presence of phenolic compounds such as ascorbic acid, gallic acid, resorcinol and quercetin in the methanolic extract of *S.hispidus DC* (stem bark). These pharmacological activities may be attributed to the various phytochemical constituents present in this extract. The purified components may have even more potency.

Further work on isolation and purification of individual groups of bioactive components can reveal the exact potential of the plant; and encourage the development of a novel broad spectrum antibacterial, antioxidant agent in future.

Conflict of Interests

The authors declare that they have no conflict of interests.

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