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Antimicrobial photochemical and proximate analysis

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Abstract
Phytochemical screening, proximate analysis and total phenolic content were also determined. Qualitative screening for phytochemical showed the presence of alkaloids, flavonoids, terpenoids, glycosides and saponins. Highest phenolic content was shown by methanol extract (49.69 mg gallic acid equivalent/g dry weight). Proximate analysis showed moisture content (3.31%), ash content (8.02%), crude fibre (1.31%), crude fat (0.97%), total protein (3.70%), total carbohydrate (86.01) and nutritive value (367.56 kcal/100 g), which would make it a potential nutraceutical. Microbial resistance to antibiotics is one of the most serious public health problems, especially in developing countries where infectious diseases still represent a major cause of human mortality.

Keywords: Antimicrobial, phytochemicals, microorganisms

Introduction
Microbial resistance to antibiotics is one of the most serious public health problems, especially in developing countries where infectious diseases still represent a major cause of human mortality. Among the microorganisms that represent a significant health threat, *Staphylococcus aureus* is highlighted as this species is responsible for a number of human illness conditions, such as skin infections and septicemia. Among fungal pathogens, the genus *Candida* also has high clinical relevance and it is responsible for a wide variety of infections, from superficial mucocutaneous to more invasive diseases. Approximately 75% of women, at least once in their life, develop candidiasis caused by *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and/or *C. krusei*. This issue encourages the search for novel antimicrobial agents. One of the oldest forms of medical practice is the use of plants for therapeutic purposes; teas, syrups, tinctures, among others have been used as medicines and in many cases come to be the sole therapeutic resource of certain communities and ethnic groups. Thus, knowledge about the therapeutic potential of plants is of great scientific and medical interest, as an effective alternative to the battle against resistant microorganisms.
Some species of the genus *Cleome* (Cleomaceae) have been investigated for medical properties and some of them had their anti-inflammatory and antimicrobial *C. spinosa* Jacq is a perennial herb that grows in the Central-West, Northeast, North and Southeast of Brazil and is known in Brazil as "Mussambê." Its leaves and flowers have been used in traditional medicine: leaves infusion is used in the treatment of asthma, cough, and bronchitis, while flowers infusion is used against fever. So far, some pharmacological actions have been proven such as antimicrobial, antinociceptive, anti-inflammatory, anthelmintic antimicrobial activity, it is only reported for essential oils from leaves, which significantly inhibit *Streptococcus pyogenes* Group A. Based on the uses of *C. spinosa* in folk medicine, it is attractive to analyze the antimicrobial potential of other tissue, using also different extraction methods. In this sense, the first step of this study was to evaluate the antimicrobial activity of different extracts from leaves and roots of *C. spinosa* against a set of 17 microbial species. The phytochemical constituents of each extract were determined and correlated with the antimicrobial action. The active extracts were tested against clinical isolates of *S. aureus* and their combinatory effects with oxacillin were also evaluated.

Phytochemical Analysis
An aliquot (10 µL) of each extract (1 mg/mL in ethanol) obtained from leaves and roots of *C. spinosa* was subjected to qualitative phytochemical analysis to ascertain the presence of

secondary metabolites such as: alkaloids, coumarins, anthracene and cinnamic acid derivatives, flavonoids, monoterpenoides, sesquiterpenoids, diterpenoids; tannins, triterpenoids and steroids (\; proanthocyanidins and leucoanthocyanidins; reducing sugars and saponins respectively. The compounds classes were visualized as aid thin layer chromatography (TLC) on silica gel 60 F254 (Merck, Germany), different systems of development and adequate visualization techniques were used: Dragendorff, NEU-PEG, KOH-Ethanol, Liebermann-Burchard, vanillin-sulfuric acid and others reagents, according to the respective method.

The solvents extraction was done by cold extraction; 50 g of dried plant was soaked in 200ml of water. The mixture was kept undisturbed at room temperature for 72 hrs in a sterile flask covered with aluminium foil to avoid evaporation and subjected to filtration through sterilized Whatman no.1 filter paper. The extracts was concentrated to dryness in rotary pressure evaporator at 40oc and stored for further antimicrobial study Test organisms used Two gram-positive organisms Staphylococcus aureus, Bacillus subtilis and two gram-negative organisms Pseudomonas aeruginosa, Escherichia coli were obtained and confirmed at the research laboratory of the Department of Microbiology, Faculty of science, University of Ibadan.

Phytochemical Screening

The aqueous extract of the plant was tested for the presence of phytochemicals qualitatively. Secondary metabolites such as phytosterols, polyphenols tannins, flavonoids), saponins, alkaloids, saponin glycosides, steroids and triterpenoids, glycosides, hydrolysable tannins, phenols and volatile oils were screened for using the procedures described by Sofowora (1993) and Harbone (1998).

Table 1: Phytochemical constituents of stem bark of Ficus thonningii

No	CONSTITUENTS	TEST	INFERENCE CE	NB	RA
	Alkaloids	Dragendorff's (Sofowora, 1993)	+	+	+
		Mayer's (Sofowora, 1993)	+	+	-
	Anthraquinones	Borntrager's (Sofowora, 1993)	+	+	+
	Carbohydrates				
	General Test	Molisch's(Sofowora, 1993)	+	+	+
	Free reducing sugar	Fehling's (Sofowora, 1993)	+	+	+
	Combined reducing sugar	Fehling's (Sofowora, 1993)	+	-	-
	Monosaccharides	Barfoed's (Sofowora, 1993)	-	-	-
	Soluble starch	(Vishnoi, 1979)	+	+	+
	Flavonoids	Ferric chloride (Trease and Evans, 2002)	+	+	-
		Lead ethanoate(Trease and Evans, 2002)	-	-	+
		Shinoda's (Trease and Evans, 2002)	+	+	+
		Sodium hydroxide(Trease and Evans, 2002)	+	+	-
	Saponins	Frothing (Sofowora, 1993)	+	+	+
	Steroids	Liebermann-Buchard's (Sofowora, 1993)	-	-	-
	Terpenoids/steroids	(Sofowora, 1993)	+	-	-
	Tannins	Ferric chloride (Trease and Evans, 2002)	+	+	+

Key = + present; - = absent; CE = crude methanolic extract; NB = n-butanol portion; RA = Residual aqueous portion

Table 2: Antimicrobial susceptibility effects of the Stem bark extracts of F. thonningii

Extract (mg per hole)	Organisms/zone of inhibition (mm) E.c	Kb	S.a	S.p	S.t	P.e
Crude 20	*a 19.33 ± 5.13	**a 26.66 ± 3.00	**a 25.33 ± 6.11	*a 32.33 ± 2.51	**a 28.33 ± 2.00	**a 33.33 ± 7.23
10	**b 11.33 ± 1.53	*a 21.66 ± 4.00	*b 18.00 ± 7.00	*a 28.00 ± 2.65	**a 18.66 ± 2.31	*a 27.33 ± 3.79
n-Butanol 20	*a 33.33 ±6.11	*a 23.00 ± 2.65	**a 26.33 ± 3.51	*a 27.66 ± 9.29	**a 31.00 ± 3.60	*a 21.00 ± 3.61
10	*a 27.00 ± 2.65	*b 18.66 ± 5.13	*b 20.00 ± 0.00	*a 22.00 ± 7.49	*b 22.66 ± 4.00	*a 15.33 ± 5.00
Aqueous layer 20	*a 30.33 ± 9.60	**a 33.33 ± 3.00	**a 17.00 ± 6.00	**a 16.66 ± 5.85	**a 30.00 ± 0.00	*a 22.00 ± 3.47
10	*b 24.00 ± 8.19	*b 26.00 ± 3.61	*b 13.00 ± 11.26	*a 11.66 ± 2.88	**a 24.33 ± 4.00	*a 16.66 ± 5.77
CIP (5 µg/disc)	* 26.67 ± 1.15	* 19.67 ± 0.58	* 19.33 ± 1.15	* 25.00 ± 0.00	* 29.33 ± 1.15	* 21.67 ± 1.15

Key: Ec = Escherichia coli; Kb = Klebsiella specie; Sa = Staphylococcus aureus; Sp = Streptococcus specie; St = Salmonella typhi; Pe = Pseudomonas aeruginosa, CIP=Ciprofloxacin, Same letter or asterisks(control) for the same portion in same column is considered insignificant (aaP>0.05, * P>0.05); different letters or asterisks on the same column is considered significant (ab$P< 0.05$, *$P< 0.05$) using paired t-test; Student-Newman-Keuls Multiple Comparisons Test; data are Mean±SD of three independent tests

Table 3: Minimum inhibitory concentration of the test extracts

	Concentration (mg ml^{-1})					
Organisms	10.0	5.0	2.5	1.25	0.125	0.625
Escherichia coli			α, ααα	αα		
Klebsiella spp.			α, αα	ααα		
Salmonella typhi			αα	α, ααα		
Streptococcus species		α	αα	ααα		
Staphylococcus aureus			α, αα	ααα		
Pseudomonas aeruginosa		α	αα, ααα			

Key: α = crude methanol extract, αα = n-butanol portion, ααα = residual aqueous portion.

Proximate analysis

The moisture, crude fibre, crude protein, ash, crude fat and carbohydrate of the sampleswas determined using methods of the Association of Official nalytical Chemists (AOAC, 1984). All determinations were done in triplicates. The proximate values were reported in percentage. Determination of moisture content was done by weighing the sample in crucible and drying in oven at 105 0C, until a constant weight was obtained, determination of ash content was done by ashing at 550 0C for about 3 hours. The kjeldah method was used to determine the protein content by multiplication of the nitrogen value with a conversion factor of 6.25. The crude fibre content of the samples was etermined by digestion method and the crude fat was done by Soxhlet extraction method. Total soluble carbohydrate was determined by the difference of the sum of all the proximate composition from 100%.

Mineral element analysis

The mineral contents of the plant seeds: potassium and sodium were determined using flame photometer, while calcium, magnesium, iron, zinc and manganese were determined using atomic absorption spectrophotometer as described the methods of the Association of Official Analytical Chemists (AOAC, 1990) after appropriate digestion by acids. All the determinations were done in triplicates.

Preliminary Phytochemical Analysis

Qualitative phytochemical analysis of the crude powder of the twelve plants collected was determined as follows: Tannins (200 mg plant material in 10 ml distilled water, filtered). A 2 ml filtrate + 2 ml FeCl3, blue-black precipitate indicated the presence of Tannins. Alkaloids (200 mg plant material in 10 ml methanol, filtered). A 2 ml filtrate + 1% HCl + steam, 1 ml filtrate + 6 drops of Mayor's reagents/ Wagner's reagent/ Dragondroff's reagent, Creamish precipitate/ Browinsh-red precipitate/ orange precipitate indicated the presence of respective alkaloids. Saponins (frothing test: 0.5 ml filtrate + 5 ml distilled water. Frothing persistence meant saponins were present). Cardiac Glycosides (Keller-kiliani test: 2 ml filterate + 1 ml glacial acetic acid + FeCl3 + conc. H2SO4). Green- blue color indicated the presence of cardiac glycosides. Steroids (Liebermann-Burchard reaction: (200 mg plant material in 10 ml chloroform, filtered). A 2 ml filtrate + 2 ml acetic anhydride + conc. H2SO4. Blue-green ring indicated the presence of terpenoids. Flavonoids (200 mg plant material in 10 ml ethanol, filtered). A 2 ml filtrate + conc. HCl + magnesium ribbon. Pink-tomato red color dicated the presence of flavonoids.

Phytochemical screening

Phytochemical analysis for antimicrobial and antioxidant compounds was one using standard methods described by (Bruneton 1999) [4] for alkaloids, nthocyanins, flavonoids, phenols, triterpenes, steroids, saponins, tannins, anthraquinones and coumarins.

Microorganisms and culture media

The microorganisms used in this study consisted of two Gram-positive bacteria *Staphylococcus aureus* ATCC25922, *Enterococcus faecalis* ATCC10541), three Gram-negative bacteria (*Escherichia coli* ATCC11775, *Pseudomonas aeruginosa* ATCC27853, *Klebsiella pneumoniae* ATCC13883) which are reference strains obtained from the American Type Culture Collection, and four clinical isolates (*Proteus mirabilis*, *Salmonella paratyphi A*, *Salmonella paratyphi B*, *Shigella flexneri*) kindly provided by the Pasteur Centre (Yaoundé-Cameroon). Three dermatophyte strains (*Trichophyton mentagrophytes* E1425, *Trichophyton terrestre* E1501, *Microsporum gypseum* E1420) were collected from "Ecole Nationale Vétérinaire d'Alford, France" and one clinical isolate (*Trichophyton equinum*) kindly provided by the Pasteur Centre (Yaoundé-Cameroon). The bacterial and dermatophyte strains were grown at 37°C and 28°C on nutrient agar (NA, Conda, Madrid, Spain) and Sabouraud Dextrose Agar (SDA, Conda) slants respectively (Tamokou *et al*. 2009).

Preparation of microbial inoculum

The inocula of bacteria were prepared from 24 h old broth cultures. The absorbance was read at 600 nm and adjusted with sterile physiological solution to match that of a 0.5 McFarland standard solution. From the prepared microbial solutions, other dilutions with sterile physiological solution were prepared to give a final concentration of 10^6 colony-forming units (CFU) per milliliter (Tamokou *et al*. 2012) [9]. Conidia suspensions of dermatophyte species were prepared from 10 days old cultures respectively.

The number of conidia was determined using a spectrophotometer and adjusted with sterile saline (NaCl) solution (0.90%) to an absorbance of 0.600 at 450 nm corresponding to a final concentration of about 1×10^5 spores/mL (Venugopal & Venugopal 1992).

Antimicrobial assay

The minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBC) and minimum fungicidal concentrations (MFCs) were determined as previously described (Tamokou *et al*. 2009; Fogue *et al*. 2012) [9].

For every experiment, a sterility check (5% aqueous DMSO and medium), negative control (5% aqueous DMSO, medium and inoculum) and positive control (5% aqueous DMSO, medium, inoculum and water-soluble antibiotics) were included. MICs were assessed visually after the corresponding incubation period and were taken as the lowest sample concentration at which there was no growth or virtually no growth. The assay was repeated thrice.

For the minimum microbicidal concentration (MMC) determination, 10 µL aliquots from each well that showed no growth of microorganism were plated on Mueller-Hinton Agar or Sabouraud Dextrose Agar and incubated at 37°C for 24 h (bacteria) and at 28°C for 10 days (dermatophytes). The lowest concentration that yielded no growth after the sub-culturing was taken as the MBCs or MFCs. Gentamycin (for bacteria) and griseofulvin (for dermatophytes) were used as positive controls.

Antioxidant assay

The free radical scavenging activity of the crude extract and fractions was evaluated as described by Tamokou *et al*. (2012) [9] with slight modifications. The test samples were prepared in methanol and 100 µL of each sample added to 900 µL of 2,2-diphenyl-1-picryl-hydrazyl-hydrate (20 mg/L DPPH) methanol solution, to give final concentrations of 50, 100, 200, 400 and 800 µg/mL. L-ascorbic acid was used as a positive control and 1mL of 20 mg/L DPPH methanol solution was used as negative control. The content of each preparation was mixed and incubated at room temperature in a dark cupboard. The absorbance was then monitored after 30 min and converted into percentage of scavenging activity:

$$\%scavengingactivity=[(Absorbanceofcontrol-Absorbanceoftestsample)/Absorbanceofcontrol]*100$$

The experiments were carried out in triplicate and the percentages of DPPH scavenged by test samples were compared to that of L-ascorbic acid. These radical scavenging percentages were plotted against the logarithmic values of the concentrations and a linear regression curve was established in order to calculate the RSa_{50}, which are the amounts of sample necessary to decrease by 50% the free radical DPPH.

Statistical analysis

Statistical analysis was carried out using Statistical Package for Social Science (SPSS, version 12.0). The experimental results were expressed as the mean ± Standard Deviation (SD). Group comparisons were performed using One Way ANOVA followed by Waller-Duncan Post Hoc test. A p value of 0.05 was considered statistically significant.

Results and Discussion

The result from the present investigation showed the presence of alkaloids, phenols, flavonoids, anthraquinones, anthocyanins, tannins and steroids in the *A. manniana* leaf extract (Table 1). These compounds varied within the fractions. In agreement with our results, the phytochemical screening of the leaf extracts of *Acalypha hispida*, *A. marginata*, *A. racemosa*, *A. monostachya*, *A. alnifolia*, *A. wilkesiana* and *A. indica* revealed the presence of phenolics, tannins, steroids, flavonoids, glycosides, saponins and anthraquinones that varied within the plant species (Iniaghe *et al*. 2009; Gotep *et al*. 2010; Canales *et al*. 2011; Evanjelene & Natarajan 2012; Mohan *et al*. 2012) [15, 12, 5, 8, 19].

The results in (Table 2) show that the methanol extract and its fractions prevented the growth of all tested microorganisms, with MIC values ranging between 0.12 and 2.04 mg/mL for bacteria, and from 0.25 to 1.02 mg/mL for dermatophytes. *S. flexneri*, *P. mirabilis* and *T. equinum* (MIC= 0.25 - 0.51 mg/mL) were the most sensitive microorganisms while *S. aureus* (MIC= 0.25 – 2.04 mg/mL) was the most resistant. The lowest MIC value for these tested samples (MIC = 0.12 mg/mL) was obtained with ethyl acetate fraction on *P. aeruginosa*. The findings of the present study explain why the plant extracts are used in traditional folk medicine and confirm the activity of *A. manniana* as a broad spectrum antimicrobial agent since it inhibited the growth of Gram-positive (*S. aureus*, *E. faecalis*) and gram negative bacteria (*E. coli*, *P. aeruginosa*, *K. pneumoniae*, *P. mirabilis*, *S. paratyphi A*, *S. paratyphi B*, *S. flexneri)* as well as some fungi (*T. mentagrophytes*, *T. terrestre*, *T. equinum*, *M. gypseum*). Similar findings had been reported for other species by Canales *et al*. (2011) [5] and Haruna *et al*. (2013) [14]. The antimicrobial activities of *A. manniana* (MIC = 0.25 - 2.04 mg/mL) can also be considered very important compared with those of Haruna *et al*. (2013) [14] who reported the antimicrobial activities of the leaves of *A. wilkesiana* methanolic extract and its four derivative fractions (MIC = 25–100 mg/mL) on human pathogenic bacteria namely *S. aureus*, *S. pyogenes*, *E. faecalis*, *P. aeruginosa*, *P. vulgaris*, *E. coli*, *Aspergillus niger*, *A. flavus*, *A. carbonerium*, *T. mentagrophytes* and *Candida albicans*. The fact that the methanolic extract of *A. manniana* and its fractions showed activity against most of the test microorganisms has added another plant in bank of herbal medicines.

Our data showed that the responses of microorganisms to tested substances varied in term of sensitivity among the strains. The differences in susceptibility may be explained by the differences in cell wall composition and/or in genetic content of plasmids that can be easily transferred among microbial strains (Karaman *et al*. 2003) [17]. It may also be explained by differences in the mechanism by which the active principles of the plant extracts exert their effect (Takeo *et al*. 2004). It was also observed that the ratios MBC/MIC found for the tested substances were generally less than or equal to 4 (Table 2), on the corresponding microbial species, suggesting that the killing effects of the extract/fractions could be expected on most of the tested micro-organisms (Tene *et al*. 2008; Tamokou *et al*. 2009). This is very interesting in view of the perspective of new antibacterial discovery from this plant, when considering the medical importance of the tested microorganisms.

Although the MIC values were ranging between 0.25 and 1.02 mg/mL for the dermatophytes, the methanol extract and fractions exerted fungistatic effects, suggesting the formation of resistance structure of dermatophytes at high concentrations (Georgopoulos & Skylakakis 1986) [10].

The results of the antioxidant activities of the methanol extract, hexane, ethyl acetate and residual fractions are presented in (Table 3). It appeared that the methanol extract and its fractions displayed important radical-scavenging activities against DPPH (RaS_{50} = 3.34 – 4.80 µg/mL) when

compared with L-ascorbic acid used as reference antioxidant (RaS_{50} = 1.74 µg/mL). These observations demonstrate that the tested samples are free radical inhibitors or scavengers acting possibly as primary antioxidants. This is very promising in the perspective of new antioxidant discovery from plant extracts. The antimicrobial activity was more concentrated in the methanol extract and contrarily, the antioxidant activity is more concentrated in the residual fraction. This suggests that *A. manniana* contains several antioxidant and/or antimicrobial principles with different polarities as shown by the phytochemical analysis. The residual fraction (Rsa50 = 3.34 µg/mL) was more active than the methanol extract (Rsa50 = 4.51 µg/mL) and hexane fractions (Rsa50 = 4.81 µg/mL) indicating that the active principles might be more concentrated in residual fraction and more diluted in others tested samples. The free radical scavenging activities of *A. manniana* (RSa_{50} = 3.34 – 4.80 µg/mL) are highest compared with those of Evanjelene & Natarajan (2012) [8] who reported the percentages of scavenging of the aqueous (91.56%), chloroform (52.55%), petroleum ether (48.34%) and methanol (88.76%) extracts of *A.alnifolia* leaves on DPPH at 0.16 mL of extracts per milliliter of solution.

Many plant extracts exhibit efficient antioxidant properties due to their phytoconstituents, including phenolics and nitrogenous compounds (Durga *et al*. 2009; Evanjelene & Natarajan 2012; Tatsimo *et al*. 2012; Tamokou *et al*. 2013) [6, 8], which were found to be present in our extracts. DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become stable diamagnetic molecules (Soares *et al*. 1997) [27]. Phenolics and nitrogenous compounds are known to be potential antioxidant due to their ability to scavenge free radicals through donation of hydrogen, forming the reduced form of DPPH. Therefore, the presence of such compounds may be responsible for the antioxidant activity found in the methanol extract and its fractions.

The methanol extract of *A. manniana* showed the highest antimicrobial activity while the residual fraction displayed the largest scavenging activity against DPPH confirming the traditional use of this plant in the treatment of various bacterial diseases such as diarrhea and skin infections. The residual fraction of *A. manniana* extract can be explored for its applications in the prevention of free radical related diseases. The obtained results might be considered sufficient for further studies geared towards the isolation and identification of the active principles and to evaluate possible synergistic effects among the extract components for their antimicrobial and radical-scavenging properties. Toxicity studies will be also necessary to establish if the crude extract/fractions could be safely used as antimicrobial/antioxidant agents.

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