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Total Phenolic Composition, Antibacterial and Antioxidant Activities of *Fagara heitzii* Aubr & Pellegr Medicinal Plant of Gabon

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ABSTRACT

Fagara heitzii Aubr. & Pellegr is a shrub indigenous to secondary forest. The plant is widely used for its various pharmacological properties. The powdered plant samples were analyzed for their phytochemical screening using standard laboratory methods. The total phenols, proanthocyanidines and antioxidant activities were evaluated with methods of the Folin-Ciocalteu, HCl-butanol hydrolysis and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. The plant is rich in phenolic compounds with tannins being the most abundant compounds in the methanolic and aqueous extracts while flavonoids and proanthocyanidins are abundant in methanol and chloroform extracts. Overall, we found that the extracts tested reduced the concentration of the free radical. The oxidation of linoleic acid was weakly inhibited by methanol and water extracts of plant (48.8 and 39.4 % ± 0.05 of reduction). In addition, the *F. heitzii* extracts had a good antimicrobial activity against tested microorganisms. In the agar-well diffusion assay, the extracts are active on both Gram positive and negative bacteria. The broth microdilution assay gave minimal inhibitory concentration values ranging from 16 to 400 µg/ml. Our results suggest that *Fagara heitzii* has both antioxidant and antibacterial activities.

Keywords: *Fagara heitzii, Antioxidant, Antimicrobial, Antiboacterial, Phenolic, Medicinal plants*

INTRODUCTION

Fagara heitzii Aubr. & Pellegr., is a large tree of secondary forest typically originated in the tropicals and subtropicals areas like in Africa. There is a variety of thorny tree with yellow-brown wood. The bark is toxic and it is used as a fish poison in many regions in Africa [1]. Rutaceae are rich in photosensitizing furanocoumarins, which are responsible for phototoxic events. Skin contact with a plant of the citrus family or one of its essential oils, cosmetics, in the presence of sunlight causes a rash, often followed by the formation of vesicles [2]. Subsequently, one can observe hyperpigmentation of the affected area due to stimulation of melanogenesis [3].

Fagara heitzii is used in decoration and used for woodworking and light for carcasses and shelves of furniture, furnishings for shops, offices and carpentry. The medicinal use of *Fagara heitzii* relies on the treatment of treat rheumatism, aches and heart palpitations, toothaches and disinfection of wounds [1]. More

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recently, phase II clinical trial fagaricine is effective against bacteria, fungi and viruses [4]. Research by Dr. ETO led to a promising discovery of the F-532 (Fagaricine 532), which is an antiviral, antibacterial and antifungal effective. The young plant of *Fagara heitzii* shoots pounded and macerated, one draws a sample whose main component is the nitidine, already well known active substance. Synergistic effects between the nitidine and its various derivatives, present in the extract obtained, give it a very interesting level of activity. It would include an anti-reverse transcriptase, classical mode of action of antivirals. Non-toxic, this remedy is now in Phase III development. The first clinical studies, shown marked decreases in viral load in subjects treated are more than encouraging. An injectable form of the drug already exists in the United States [4].

The present study reports results of a detailed analysis of the antioxidant and antibacterial activities of aqueous and methanolic extracts of *Fagara heitzii*. To the best of our knowledge, such investigation has not been conducted yet on *F. heitzii* species from Gabon.

Materials and Methods

Plant materials

The leaves of *Fagara heitzii* were selected according to their traditional uses. The plant samples were collected in Oyem (Northern of Gabon) in September 2012. Identification of the species was carried out at the National Herbarium of IPHAMETRA, Libreville (Gabon). Voucher specimens have been deposited in the Herbarium of IPHAMETRA and at Laboratoire de Substances Naturelles (LASUNA) at the Department of Chemistry-Biochemistry, faculty of sciences of USTM in Franceville.

Compounds extraction and qualitative analysis

The harvested samples were dried in the laboratory at room temperature and subsequently pulverized with a mechanical crusher. For aqueous extraction, 50 g of powder were percolated for 24 h in 300 ml water. After cooling at room temperature, the extract was filtred and lyophilised. For methanolic extraction, 50 g of powder were percolate in 500 ml of methanol for 24 h. For chloroform extract, 50 g of powder were percolated with 500 ml of chloroform for 24 h. Methanol and chloroform were evaporated with a rotary evaporator. The extract was then washed with hexane in order to eliminate the chlorophyll and other pigments. The solvent were evaporated and the extract lyophilised.

Stock solutions were prepared by dissolving lyophilized extracts in distilled water for aqueous extracts and in a of dimethyl sulfoxide (DMSO) for chloroform and methanol extracts. The final concentration of DMSO was set at 0.5 % after a two-fold dilution of the extracts from 2000 to 400 µg/ml.

Each extract was then tested for the presence of saponosids, reducing compounds, phenolic compounds, flavonoids, proanthocyanidins, sterols, triterpens, carotenoids, tannins and anthracenosids as described elsewhere [5].

Phenolic content: The Folin-Ciocalteu method was used to measure total amount of phenolic content [6]. The original assay was adapted to a microtiter 96-wells plate system [7] and gallic acid (3, 4, 5-trihydroxybenzoic acid) was used as standard. To 20 µl of beverage 80 µl of Folin-Ciocalteu reagent were added. After 5 min of incubation at room temperature (20°C), 80 µl of 20 % (w/v) sodium carbonate solution was added and incubated. The mix was then incubated for 30 min and the absorbances read at 760 nm. All tests were carried out in triplicate and results were expressed as gallic acid equivalent (GAE).

Tannins: The reference method of european communauty was used to measure total amount of tannins (1994) [8].

Proanthocyanidins (PAs) were quantified with an adaptation to a 96-well plate assay [9, 10]. The method consists on the hydrolysis of proanthocyanidins in a hot acid-alcohol medium into anthocyanidins. This method allows taking into account all the units of flavans-3-ols constituting the polymers [11]. The assay is performed by mixing 50 µl of the extract with 700 µl of 30 % HCl-butanol solution (v/v). The mixture was put in tightly closed 1.5 ml Eppendorf tube and vortexed for 1 min. Subsequently, the tube was heated at 100°C for 2 h and after cooling, 200 µl aliquots were put in triplicate into a 96-well plate and the absorbances were read at 550 nm. Apple procyanidins (DP ≈ 7.4) treated as aforementioned were used as a standard. Results were expressed as apple procyanidins equivalent (APE).

Flavonoids : The Dowd method was used to measure total amount of flavonoids [12]. The original assay was adapted to a microtiter 96-wells plate system [7]. Quercetin was used as standard. 100 µl of 2 % $AlCl_3$ in methanol were added to 100 µl of plant extract. After 10 min of incubation at room temperature (20°C), the absorbances were read at 415 nm. All tests were carried out in triplicate and results were expressed as qercetin equivalent (QE).

Microorganisms, antibiotics and media

Commercially available antibiotics discs, Penicillin 10 IU/IE/UI, Tetracyclin 30 µg and Spectinomycin 100 µg were purchased from Beckton Dickinson. All media used were from Oxoid. Microorganisms included reference strains and fresh clinical isolates. The selection of clinical microorganisms depended on their availability, thus microorganisms that have been reported to be the most frequently implicated in infectious diseases in tropical areas were well represented [13-15].

Clinical isolates were *Staphylococcus aureus* (n = 5), *Enterococcus faecalis* (n = 3), *Pseudomonas aerugenosa* (n = 5), *Salmonella enterica* (n = 5) and *Streptococcus pyogenes* (n = 5). All these strains were isolated from clinical samples at Laboratoire de Biochimie du Centre Médical de l'USTM, Franceville.

The microorganisms were identified by the use of their biochemical profiles as recommended by the manual "Bactériologie Médicale" [16]. The reference strains were *Escherichia coli* CIP 105182, *Enterococcus faecalis* CIP 103907, *Bacillus cereus* LMG 13569 BHI, *Listeria innocua* LMG 135668 BHI, *Staphylococcus aureus* ATCC 25293 BHI, *Staphylococcus camorum* LMG 13567 BHI, *Proteus mirabolis* CIP 104588, *Shigella dysenteria* CIP 5451 and *Staphylococcus aureus* ATCC 9144.

Antibacterial assays

Agar-well diffusion: The assay was conducted as described by Perez et *al.* (1991) [17]. Briefly, microorganisms from growth on nutrient agar incubated at 37 °C for 18 h were suspended in saline solution 0.9 % NaCl and adjusted to a turbidity of 0.5 Mac Farland standards (10^8 cfu/ml) [18]. The suspension was used to inoculate 90 mm diameter Petri plates with a sterile non toxic cotton swab on a wooden applicator. Six millimeters diameter wells were punched in the agar and filled with 50 µl of 2000 µg/ml plant extract. The dissolution of the extract was added by 0.5 % (v/v) DMSO which did not affect microorganism growth, according to our control experiments. Commercial antibiotics were used as positive reference standard to determine the sensitivity of the strains. Discs were directly placed onto the bacterial culture. Plates were incubated in air at 37 °C for 24 h. Antibacterial activities were evaluated by measuring inhibition zone diameters (IZD).

Broth microdilution assay: Broth microdilution method was used to determine minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of the extract against the test microorganisms as recommended by the National Committee for Clinical Laboratory Standards [19, 20]. The tests were performed in 96 well-plates. Extracts were dissolved in 0.5 % DMSO was transferred in plates to obtain a two-fold serial dilutions ranging from 3.2 to 400 µg/ml. Then plates were inoculated with microbial suspensions diluted from the same 0.5 Mac Farland standards to have 10^8cfu/ml in each well [18]. The final volume in wells was 200 µl. After 24 h incubation in air at 37 °C, MIC was recorded as a lowest extract concentration demonstrating no visible growth in the broth. MBC was recorded as a lowest extract concentration that kills 99.9 % of bacterial inocula. MBC values were determined by removing 100 µl of bacterial suspension for subculture demonstrating no visible growth and by inoculating nutrient agar plates. Plates were incubated at 37 °C for a total period of 48 h [14, 15].

Antioxidant activity

Determination of DPPH radical scavenging activity: The free radical scavenging activity of chloroform and methanol extracts were determined according to the method described by Burits and Bucar (2000) [21]. Experiments were carried out as described previously [14, 15, 22]. Briefly, 0.5 mM DPPH (Fluka) radical solution in methanol was prepared, and then 1 ml of this solution was mixed with 3 ml of the sample solution in ethanol. Various concentrations of extracts were obtained. BHT (Sigma) was used as a positive control at 100 g/ml concentration. After incubation for 30 min in the dark, the absorbance was measured at 517 nm. Decrease in the absorbance of the DPPH solution indicates an increase in DPPH radical scavenging activity. This activity is given as percent DPPH radical scavenging, which is calculated with the following equation [9, 10]:

% DPPH radical scavenging = 100 x $[(A_{control} - A_{sample})]/A_{control}$.

The control contained 1 ml of DPPH solution and 3 ml of ethanol. The measurements of DPPH radical scavenging activity were carried out for three sample replications and values are an average of three replicates.

Determination of antioxidant activity: The anti oxidant activity was determined according to the method described by Dapkevicius et *al.* (1998) [23]. 0.5 mg of -carotene was dissolved in 1 ml of chloroform (HPLC grade); 25 µl of linoleic acid and 200 mg of tween 40 were added as emulsifier because carotene is not water soluble. Chloroform was completely evaporated using a vacuum evaporator. Then, 100 ml of distilled water saturated with oxygen was added with vigorous shaking at a rate of 100 ml/min for 30 min; 2500 µl of this reaction mixture was dispersed into test tubes, and 350 µl of 2 g/l portions of the extracts, was added. The emulsion system was incubated for up to 48 h at room temperature. The same procedure was repeated with a positive control BHT and a blank. After this incubation time, the absorbance of the mixture was measured at 490 nm. Antioxidant capacities of extracts were compared with those at the BHT and the blank. Tests were carried out in triplicate [14, 15]. The Relative Antioxidant Activity (RAA %) of the extracts was calculated from the equation:

RAA % = $(A_{sample}/A_{BHT}) \times 100$, where A_{BHT} is the absorbance of the positive control BHT and A_{sample} being the absorbance of the extract.

Statistical analysis

Data were expressed as mean ± SEM. A one way variance was used to analyze data. $P < 0.01$ represented significant difference between means (Duncan's multiple range test).

Results and Discussion

Phytochemical screening

The phytochemical screening of the extracts was first performed to detect the major chemical groups present in the extracts. The results of this screening are shown in Table 1.

The methanolic extracts contained the most detected chemical groups compared to water or chloroform extract. Overall, we found that extracts of *Fagara heitzii* are rich in phenolic compounds. The tannins are abundant in methanolic and aqueous extracts. Chloroform and water extracts are rich in phenolic compounds and reducing compounds while flavonoids and proanthocyanidins are abundant in methanol

Table 1: Chemical groups detected in *Fagara heitzii* extracts

Extracts	Water	Chloroform	Methanol
Saponosids	+++	-	-
Tannins	++	+	+++
Reducing compounds	+++	+++	-
Phenolic compounds	+++	++	-
Flavonoids	-	++	++
Proanthocyanidins	-	++	++
Anthracenosides	-	-	-
Sterols and triterpens	-	-	-
Carotenoids	+	-	+++
Bial reaction	-	+++	++
Foulger reaction	-	+++	++
Polyphenols	+++	-	++

-: Not detected, +: Rare, ++: Abundant, +++: Very abundant

Table 2: Antioxydant activity of Fagara heitzii Aubr. & Pellegr.

Extracts			Water	Chloroform	Methanol
	Yield of extraction (%)		4.6	1.79	0.5
Phenolic Content	Phenolic compounds (µg GAE/ml)		1094.67	266.75	nd
	Flavonoids (µg QE/ml)		106.74	693.33	615.62
	Proanthocyanidins (µg APE/ml)		339.83	1836.56	639.34
	Tannins (µg TAE/ml)		732.82	116.33	1385.41
Antioxidant activity	DPPH	IC_{50} (µg /ml)	0.21	nd nd	0.22
	β-carotène	RAA (%)	48.8±0.5		39.4±0.5

nd : not determined

and chloroform extracts. The methanol extract is rich in carotenoids (Table 1).

Antioxidant activity

We further determined the antioxidant activity (Table 2). The results of these tests are given in Table 2. The yield of aqueous, chloroform and methanol extracts of *Fagara heitzii* were respectively 4.6 % (v/v), 1.79 % (v/v) and 0.5 % (v/v).

Levels of phenolic content were expressed in terms of gallic acid equivalent (GAE). The equation of the right and side of the proportioning of total phenolic content by the method of Folin-Ciocalteu gave Y = 0.0012 X – 0.0004 with R^2= 0.9902 [24]. The total contents of phenols ranged between 1094.67 µg GAE/ml and 266.75 µg GAE/ml of aqueous and chloroformic extracts (Table 2). The HCl/butanol assay used here for the determination of proanthocyanidins is more specific than many other tests such as the vanillin assay [9, 10]. Levels of proanthocyanidins were expressed in terms of apple procyanidins equivalent (APE). The equation of the right-hand side of the proportioning of the proanthocyanidins by the HCl- Butanol method gave Y = 0.0006 X + 0.0024 with R^2 = 0.9869 [24]. Proanthocyanidins were 339.83 µg APE/ml, 1836.56 µg APE/ml and 639.34 µg APE/ml of aqueous, chloroformic and methonlic extracts. Levels of flavonoids were expressed as quercetin equivalent (QE). The equation of the right-hand side of the proportioning of the qercetin by the Dowd method gave Y = 0.0032 X + 0.0077 with R^2 = 1 [2]. Flavonoids were 106.74 µg QE/ml, 693.33 µg QE/ml and 615.62 µg QE /ml of aqueous, chloroformic and methonlic extracts. Levels of tannins were expressed in terms of tannic acid equivalent (TAE). The equation of the right-hand side of the proportioning of the total tannins by the reference method of

Table 3: Inhibition zone diameters (IZD) recorded in agar diffusion with of *Fagara heitzii* extracts

Reference strains	Diameters of inhibition zone: IZD (mm)					
	Gram	MeOH	Chl	P	TE	SPT
E. coli CIP 105182	-	13	12	nd	nd	20
Shigella dysenteria CIP 5451	-	20	13	nd	16	24
Proteus mirabolis CIP 104588	-	nd	nd	7	15	27
Enterococcus faecalis CIP 103907	+	nd	15	27	19	36
Bacillus cereus LMG 13569 BHI	+	19	10	21	18	21
Listeria innocua LMG 135668 BHI	+	11	13	27	14	21
Staphylococcus aureus ATCC 25293 BHI	+	nd	nd	23	17	17
Staphylococcus camorum LMG 13567 BHI	+	13	17	33	21	25
Staphylococcus aureus ATCC 9144	+	13	21	19	26	21
Clinical isolates						
Pseudomonas aerugenosa (n=5)	-	9±1	nd	9±1	21±4	28±3
Salmonella enterica (n=5)	-	20±2	24±3	nd	16±2	27±3
Enterococcus faecalis (n=3)	+	nd	13±2	24±3	17±2	26±2
Staphylococcus aureus (n=5)	+	13±2	21±3	39±3	nd	16±2
Streptococcus pyogenes (n=5)	+	7±1	13±2	29±2	21±2	30±3

nd : not determined ; Chl : chloroform extract; MeOH : methanol extract; Commercially available antibiotics discs, P: Penicillin; TE: Tetracyclin and SPT :Spectinomycin.

European Community (1994) gave Y = 0.0009 X + 0.2088 with R^2=1. Among *Fagara heitzii* extracts, tannins contents were 732.82 µg TAE/ml (water), 116.33 µg TAE/ml (chloroform) and 1385.41 µg TAE/ml (methanol). The concentration of proanthocyanidins and flavonoids is greater in chloroform extract while the tannins content is greater in the methanol extract and total phenols in aqueous extract.

For the DPPH test, extracts reduced the concentration of the free radical. The antioxidant capacity of extracts is significantly lower than the positive control BHT ($p > 0.01$), but significantly higher than the negative control. The IC_{50} of *Fagara heitzii* is 0.21 ± 0.1 mg / ml. The oxidation of linoleic acid is weakly inhibited by methanolic and aqueous extracts of *Fagara heitzii* (RAA=48.8 and 39.4 ± 0.05 %). The capacity of the methanol extract to reduce the β-carotene is greater than that of the aqueous extract and does not exceed 50 % in both cases. The antioxidant activity does not follow a radical process. What makes these extracts *Fagara heitzii* low potential antioxidant. The results show that the bark of *Fagara heitzii*, contains extractable polar compounds more abundant than the non-polar compounds. The concentration of flavonoids and

tannins is greater in the methanol extract compared to water extracts and chloroform while those of total phenols and proanthocyanidins are respectively higher in water extracts and chloroform. This activity is due to the chemical composition of extracts poor in polyphenols strongly hydroxylated.

Diameters of inhibition zone by disc assay of *Fagara heitzii*.
Susceptibility testing of bacterial and fungal strains to extracts *Fagara heitzii* gave values of inhibition zones (Table 3). The antimicrobial screening of extracts of *Fagara heitzii* shows that they have a potential inhibitor. The extracts are active on both Gram positive and negative bacteria. Methanol and chloroform extracts gave a high antimicrobial activity and a significant inhibition with diameters ranging from 12 to 24 mm. *Salmonella enterica* strains (24 mm), *Staphylococcus aureus*, *Staphylococcus aureus* ATCC 9144 (21 mm) were most sensitive to the chloroform extract. This extract gave antibacterial activity through inhibition with diameters of 13-17 mm *Staphylococcus camorum* LMG 13567 BHI (17 mm), *Enterococcus faecalis* CIP 103907 (15 mm), *Listeria innocua* LMG 135668 BHI, *Shigella dysenteria* CIP 5451, *Enterococcus faecalis and Streptococcus pyogenes* (13 mm).

The methanol extract yielded the zones of inhibition ranging from 9 to 20 mm. This extract has the strongest growth inhibition activity on *Salmonella enterica*, *Shigella dysenteria* CIP 5451 (20 mm) and *Bacillus cereus* LMG 13569 BHI (19 mm). Its gave an intermediate antibacterial activity with inhibition diameters of 13 mm on *E. coli* CIP 105182, *Staphylococcus aureus* ATCC 9144, *Staphylococcus aureus* (13 mm). *Enterococcus faecalis* CIP 103907, *Enterococcus faecalis, Listeria innocua* LMG 135668 BHI and *Streptococcus pyogenes* showed resistance to the methanol extract whereas strains *Proteus mirabolis* CIP 104588, *Staphylococcus aureus* ATCC 25293 BHI and *Pseudomonas aeruginosa* were not inhibited by chloroform and methanol extracts.

The methanol extract is active and has bactericidal properties on the and wild and reference bacterial strains studied with MIC and MBC equal to 25 mg/ml of *Enterococcus faecalis*. The methanol extract is bacteriostatic against *Enterococcus faecalis* CIP 103907. The other strains are sensitive and the extract is bactericidal with MIC and MBC equal to 50 mg / ml of *E. coli* CIP 105182, *Bacillus cereus* LMG 13569 BHI, *Listeria innocua* LMG IHB 135668, *Staphylococcus aureus* ATCC 25293 BHI, *Proteus mirabolis* CIP 104588, *Staphylococcus camorum* LMG 13567 BHI, *Shigella dysenteria* CIP 5451, *Staphylococcus aureus* ATCC 9144, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Pseudomonas aerugenosa*.

Table 4: MIC and MBC values of *Fagara heitzii* (mg / ml), the microdilution assay

Extracts	Chloroform		Methanol	
Reference strains	**MIC**	**MBC**	**MIC**	**MBC**
E .coli CIP *105*182	50	50	50	50
Enterococcus faecalis CIP 103907	>100	nd	25	100
Bacillus cereus LMG 13569 BHI	100	50	50	50
Listeria innocua LMG 135668 BHI	25	25	50	50
Staphylococcus aureus ATCC 25293 BHI	25	25	50	50
Proteus mirabolis CIP 104588	25	25	50	50
Staphylococcus camorum LMG 13567 BHI	100	100	50	50
Shigella dysenteria CIP 5451	100	100	50	50
Staphylococcus aureus ATCC 9144	100	100	50	50
Clinical isolates				
Staphylococcus aureus (n=5)	100	nd	50	50
Streptococcus pyogenes (n=5)	>100	nd	50	50
Enterococcus faecalis (n=3)	50	50	25	25
Pseudomonas aerugenosa (n=5)	>100	nd	50	50
Salmonella enterica (n=5)	100	50	>100	nd

Broth microdilution assay
The MICs and MBCs of extracts *Fagara heitzii* on bacterial strains are shown in Table 4.

The highest bactericidal activity of the chloroform extract was recorded on strains *Listeria innocua* LMG 135668 BHI, *Staphylococcus aureus* ATCC 25293BHI and *Proteus mirabolis* CIP 104588. This extract is bactericidal with MIC and MBC equal to 50 mg / ml of *E. coli* and *Enterococcus faecalis* CIP 105182, and 100

mg / ml of *Staphylococcus camorum* LMG 13567 BHI, *Shigella dysenteria* CIP 5451, *Staphylococcus aureus* ATCC 9144.

Conclusion

Fagara heitzii Aubr. & Pellegr., is rich in phenolic compounds displayed good antimicrobial activity against several test microorganisms. The extracts reduce the concentration of the free radical. The results of the present study support the traditional medicinal use of *Fagara heitzii* and suggest that a great attention should be paid to this plant which is found to have many pharmacological properties. The study represents the antimicrobial and antioxidant activities of the crude extracts of the plant. Further investigations are undergoing for the isolation and identification of active principles.

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