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Evaluation of the phytochemical composition, antimicrobial and anti-radical activities of *Mitracarpus scaber* (Rubiaceae)

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Mitracarpus scaber **is a medicinal plant used in traditional practices for the treatment of dermatoses and liver diseases. The objective of this study was to quantify the content of phenolic compounds and to evaluate the anti-radical and antimicrobial activities of four types of its total extracts on ten microbial strains. The Folin-Ciocalteu method was used to determine total phenol content, condensed tannin content by the Butanol-HCl method and anti-radical activity by reduction of phosphomolybdate. The micro-dilution technique coupled with spreading in an agar medium made it possible to evaluate the antimicrobial activity. The results obtained showed that the total phenol content varies according to the nature of the extracts and ranges from 36.75±1.62 mg / g to 14.63±0.44 mg / g of extract. The contents of condensed tannins ranged from 41.83%±0.03 mg CE / g to 0.39%±0.14 mg CE/g. The anti-free radical activity was between 0.48±0.06 mg AAE / g and 0.21±0.00 mg AAE/g. The antimicrobial activity gave MIC of 6.25 to 50 mg / ml. The hydroalcoholic extract showed lower MIC and would therefore be best suited for the treatment of microbial diseases.**

Key words: *Mitracarpus scaber*, antimicrobial activity, antiradical activiy, phenols.

INTRODUCTION

Plants in their diversity are a gift of nature to man. They contain a lot of important molecules that justifies their therapeutic use in traditional medicine. The number of plant species was estimated to be about 400,000 to 500,000 (Karou et al., 2006). The use of plants in therapeutic care was known to all peoples. Even today, they continue to prove themselves especially in countries where low-income indigenous populations do not have access to modern medical care. Thus, in African traditional medicine, *Mitracarpus scaber,* an annual tropical plant of Rubiaceaes family about 10 to 50 cm high (Nathalie, 2002) with rough leaves (Olorode et al., 1984) was used. At maturity, this plant makes white flowers at the level of each armpit of the leaves. *M. scaber* grows on degraded soils in Africa and Asia (Moussa et al., 2015). It was also found in Latin America (Yaméogo, 1982). In Togo, *M. scaber* can be harvested from June to November. In Togolese traditional medicine, the plant was used to treat infected wounds, skin (Magbefon et al., 2009). It was also used orally in combination with sesame to treat liver problems. Similarly, in several other countries in Africa, it was known and

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used to treat dermatoses, headaches, toothache, amenorrhea, dyspepsia, venereal diseases (Kerharo and Adam, 1974). As a result, these traditional practices have prompted researchers to conduct scientific study to verify many therapeutic effects that *M. scaber* is said to have. For this reason, several extracts of the plant have been studied in order to test its antimicrobial, antifungal activities and even its hepato-protective effect. At the same time, other researchers have been interested in the qualitative chemical composition of plant extracts. This study was undertaken to contribute to the evaluation of its phytochemical constituents and also, to explore the anti-radical and antimicrobial potentials of its various extracts.

MATERIALS AND METHODS

Plant material

The whole plant of *M. scaber* was harvested.

Solvents

The solvents used include the following: Chloroform, petroleum ether and methanol.

Culture media and reagents

To carry out extractions and phytochemical tests, the following reagents were used: 95° ethanol, methanol, Merck's Folin-Ciocalteu reagent, butanol, hydrochloric acid, gallic acid, PROLABO ascorbic acid, Sodium carbonate, ammoniacal iron sulfate, sulfuric acid, sodium phosphate and ammonium molybdate from PROLABO. Muller Hinton agar from MAST House and Liofichem nutritious as well as Muller Hinton Broth from MAST House were used for antimicrobial testing.

Microbial strains

Antimicrobial tests were performed with reference strains: *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Salmonella* OMB; wild strains of *Staphylococcus aureus*, *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* ATCC 27853, *Citrobacter diversus* and on *Candida albicans* provided by Laboratory of Microbiology and Quality Control of Foodstuffs (LA-MI-CO-DA).

Sample collection

Fresh whole plant of *M. scaber* was collected between June and November 2015 in Kozah at 400 km north of Lomé (Togo). The plants was identified and confirmed at the Herbarium of Department of Plant Biology, Faculty of Sciences, University of Lomé (FDS-UL).

Preparation of extracts

The plant materials (fresh leaves) was dried in the laboratory (LASEE-UK) at room temperature (25°C) and pulverized into a fine powder for extraction by a Moulinex brand of Binatone. Several extracts were prepared from the powder obtained. The procedure was carried out using water, ethanol-water (70:30 v/v) methanol and organic solvent such as chloroform.

Hydroalcoholic extraction

100 g of the powdered plant materials was extracted using percolation process in 500 ml distilled water- ethanol (30:70).

Aqueous extraction

For aqueous extraction, 100 g of powder was mixed in 500 ml of distilled water.

Chloroform extraction

Chloroform extract was prepared adding 100 g of powder to 500 ml of chloroform.

Methanol extraction

For methanol extract, 100 g of powder was dilapidated with petroleum ether before adding to 500 ml of methanol using percolation process overnight. Each mixture was subjected to continuous stirring by an orbital stirrer for 48 h. At the end of the stirring, the mixture was decanted and filtered on Wattman paper. The filtrate was evaporated to dryness to obtain residue in vacuole using a Heidolph Laborata 4000 rotavapor at 60°C.

Evaluation of total polyphenol content

The total polyphenols content was determined according to the Folin-Ciocalteu (FCR) method described by Karou et al. (2006). For this test, the standard curve was prepared using garlic acid; linear-dose-response regressing curve was generated at absorbance of 760 nm with a UNICO model 12 spectrophotometer against a negative control consisting of a mixture of 0.5 ml of FCR, 0.5 ml of sodium carbonate, distilled water and a positive control consisting of extract and distilled water.

Estmation of proanthocyanidol content

The proanthocyanidol content was evaluated by the method of Butanol-HCl, developed by Porter et al. (1986). The test consisted of mixing 0.2 ml of each extract with 0.2 ml of ammoniacal iron sulfate (20 g/L) and 7 ml of a solution of butanol / hydrochloric acid (95/5 ml) in the tubes. After 40 min incubation in a water bath at 95°C, the tubes were cooled to room temperature and their absorbance read at 540 nm. The concentration of proanthocyanidin extracts was obtained by the following relationship developed by Aboh et al. (2014):

$X = (\text{Absorbance} \times 1 \text{ CE/g}) / 0.280$
Absorbance = optical density of extract measured at 540 nm;
CE = equivalent catechin).

Evaluation of the anti-radical activity of the extracts by the phosphomolybdate reduction method. The reduction of the phosphomolybdate was carried out according to the method described by Prieto et al. (1999) and Karou et al. (2006). 1 ml of each extract was added to 9 ml of reagent (phosphomolybdate)

Table 1. Content of total phenols, proanthocyanidols and antiradical compounds.

Components extracts	Total polyphenols (mg AGE/g)	Proanthocyanidols (mg CE/g)	Antiradical component (mg AAE/g)
EM1	31.58±2.42	1.83%±0.03	0.48 ±0.06
EM2	25.74±0.09	1.59%±0.03	0.44 ±0.04
EC	14.63±044	0.39%±0.14	0.21±0.00
EH	19.13±1.32	0.61%±0.01	0.29±0.01
EA	36.75±1.62	1.01%±0.15	0.28±0.00

EM1 non-delipidated methanol extract; EM2 delipidated methanol extract; EC chloroform extract; EH hydroalcoholic extract; EA aqueous extract; Mg AGE/g milligram gallic acid equivalent per gram of extract; Mg CE/g milligram catechin equivalent per gram of extract; Mg AAE/g milligram equivalent ascorbic acid per gram of extract.

and the whole was heated at 95°C for 90 min in a water bath after which the mixture obtained was cooled to room temperature. Ascorbic acid was used as a standard antioxidant under the same experimental conditions. The results were expressed in milligrams of equivalent of ascorbic acid per gram of crude extract.

Antibiogram of the germs studied

A bacterial suspension was prepared in sterile distilled water from pure culture of 24 h from nutrient agar. This suspension was compared to the standard of the Mc Farland 0.5 solution which corresponds to 108 CFU/ml. The suspensions thus obtained were seeded by swabbing on Mueller Hinton agar. The thickness of the (Comité de l'Antibiogramme de la Société Française de Microbiologie, 2015). The antibiotic discs were placed on Petri dishes. After 10 to 15 min, the plates were incubated at 37°C for 24 h. The diameters of the zones of inhibition were measured and compared with sensitive one.

Microbicidal activity of extracts

Minimal Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) were determined using the microdilution technique in 96-well plates with Muller Hinton broth (MHB) (Clinical and Laboratory Standards Institute, 2012). To this end, stock solutions of the total extracts sterilized by millipore membrane filtration of 0.45 µm in diameter were prepared at 100 mg/ml in an ethanol / water mixture (10/90). One hundred microliters (100 µl) of MHB were deposited in the wells of the microplate and successive dilutions of 100 to 12.5 mg/ml of extract were made with BMH. Microbial turbidity suspensions corresponding to Mac Farland 0.5 were made with microorganisms from a 24-h culture at 37°C after a control Gram. Then, 100 µl of this microbial suspension was brought into contact with the extracts and their dilutions in the wells. Positive control wells (microbial suspension without extract) and negative controls (MHB + extract) were also formed. Gentamicin was used as a reference antibiotic. Finally, the plates were stirred and incubated at 37°C for 24 h. The MBC was determined by taking 100 µl of suspension in the wells without visible growth and by seeding on the nutrient agar. Incubation was carried out at 37°C for 24 to 48 h at the end of which the colonies were counted.

Bactericidal kinetics

For this test, the ten strains studied were tested by a single concentration of MIC of the hydroalcoholic extract. Thus, 100 µl of each microbial suspension (108 CFU/ml) of MHB was brought into contact with 100 µl of extract (100 mg/ml) at initial time (t = 0). Samples of 100 µl were plated on nutrient agar at t = 0 and after incubation times of 15, 30, 45 min and 24 to 48 h for certain germs. The dishes were incubated at 37°C and the colonies were counted in 24 h. Control microbial suspensions without extract were made.

Statistical analysis

The statistical analyses were carried out using Epi-info version 6.04 dfr. The parametric analyses were performed by the ANOVA (Variance Analysis) test. The difference between the averages is considered statistically significant at the 5% threshold ($P < 0.05$).

RESULTS AND DISCUSSION

Content of total polyphenols, proanthocyanidols and antiradical activity of extracts of *M. scaber*

Quantitative chemical analyses were carried out on the total extracts of the powder of the plant studied. The results indicated that the aqueous extract was richer in total phenols (36.75 ± 1.62 mg / g of extract). The chloroform extract had the lowest phenol value (14.63 ± 0.44 mg / g extract); while the methanolic extracts were rich in condensed proanthocyanidols or tannins (1.86% CE/g extract for the non-dilapidated methanolic extract followed by the dilapidated methanol extract, 1.59% CE/g). The results are recorded in Table 1.

Studies were carried out on *M. scaber*. There are limited quantitative studies on the phenolic compounds of the total extracts of the plant in Nigeria. Aboh et al. (2014) studied the phenolic qualitative composition of *M. scaber* using diethyl ether and tannins with ethyl acetate. It appears from their study that the said plant had a content of 9% of phenols and 1.4% of tannic compounds. Methodological, soil and climatic differences could explain the differences between the results obtained.

Koudoro (2015), on several plant extracts, also showed that the aqueous extracts had the highest total phenol contents than the other solvents. The differential solubility

Table 2. Sensitivity of microbial strains studied to antibiotics.

Germs \ Antibiotics	Lincomycine	Penicilline G	Norfloxacine	Nétilmicine	Tobramycine	Ceftriaxone
E. coli	R	R	R	S	S	S
E. coli ATCC 25922	R	R	S	S	S	S
S. aureus	S	I	I	S	S	S
S.aureus ATCC 29213	R	I	I	S	S	S
S. aureus ATCC 25922	S	I	I	S	S	S
P. aeruginosa ATCC 27853	R	R	I	S	S	S
Salmonella OMB	R	R	I	S	S	S
K. pneumoniae	R	R	S	S	S	S
C. diversus	R	I	S	S	S	S
C. albicans	S	I	I	S	S	S

S: Sensitive; R: Resistant; I: Intermediate.

of total phenols in solvents was explained by the PH and the polarity of these molecules and solvents. Other studies have shown that phenolic compounds possess antioxidant activities (Karou et al., 2006; Koudoro, 2015).

The present study evaluated the anti-radical activity of *M. scaber* and the reduction of molybdate VI to molybdate V in hot and acid medium. The results of this quantitative test show that there was a good correlation between the contents of the proanthocyanidol and the measured values of the antiradical activity (r2 = 0.92). The high value was obtained with methanol extract (0.48 mg AA/g extract) and the low value was obtained with the chloroform extract (0.21 mg/g extract). In this context, the antiradical activity of methanol extract was evaluated with the DPPH method. At the end, 50% effective concentration (EC50 of 41.64 ± 1.5 µg/ml) was obtained (Germano et al., 2000). Anti-free radicals were substances that could neutralize or reduce the damage caused by free radicals in the body. Thus, the use of M. scaber in cosmetic products and in liver therapy could therefore be justified by the above results. The results of the statistical analyses (total phenols = 0.000501, P proanthocyanidols = 0.001311, antiradical activity = 0.020491) showed that the content of total phenol extracts, proanthocyanidins and antiradical compounds depends on the solvents nature (P <0.05).

Test of sensitivity of strains studied to conventional antibiotics

The results of this test are shown in Table 2. All of the Gram-negative microbial strains tested were resistant to Lincomycin including the reference strain S. aureus ATCC 29213. Similarly, all enterobacteria resisted the action of Penicillin G. E. coli strain was sensitive to three antibiotics tested (Tobramycin, Netilmicin and Ceftriaxone). While the *E. coli* ATCC strain 25922 in addition to these three was sensitive to Norfloxacin. Any antibiotics inhibited in vitro all of organisms tested growth. The resistance of the tested microbial strains to antibiotics would be linked to several factors. Indeed, to be active, an antibiotic must first enter the bacterial cytoplasm, without being modified.

Other antibiotics act either by inhibiting nucleic acid synthesis of the bacteria or by disrupting the cytoplasmic membrane or by disrupting bacterial proteins or by acting on membrane permeability or by acting on the intermediate metabolism (Marjorie, 2007). Despite these various mechanisms of action, microorganisms were also endowed with mechanisms of resistance that were natural or acquired. For example, Gram-negative bacilli (*E. coli*, Salmonella sp., *P. aeruginosa* etc.) were naturally resistant to hydrophobic antibiotics. The bacterium can modify the point of attachment of the antibiotic and thus become resistant. Some antibiotics pass through the outer membrane of the wall of these bacteria with difficulty because of the presence of lipids. Lincomycin belongs to lincosamides and Penicillin of first-generation β lactams. These two molecules were comparable in spectrum of action. They were ineffective on Enterobacteriaceae and on genus of Pseudomonas (Anne, 2014).

E. coli strain was resistant to Norfloxacin which was a fluoroquinolone and targeted at bacterial DNA. This result could be explained by a resistance acquired by *E. coli* strain by transfer of plasmid or chromosomal mutation. Ceftriaxone belongs to the third-generation of cephalosporins. The susceptibility of the strains was due to the lack of production of cephalosporinases of the strains or to an ineffective production of this enzyme in the face of the antibiotic. Similarly, the microbial strains studied were sensitive to Tobramycin and Netilmicin, which were water-soluble and positively charged aminosides (Anne, 2014).

Evaluation of antimicrobial activity of total extracts of *Mi. scaber*

The antimicrobial tests carried out with the five extracts

Table 3. Antibacterial activity of total extrats of *M. scaber.*

Microbial strains / Extracts/ Concentrations (mg/ml)	*Candida albicans*	*S. a*	*S. a* ATCC 25923	*S. a* ATCC 29213	*E. c*	*E. c* ATCC 25922	*P. a* ATCC 27853	*S. OMB*	*K. p*	*C. d*
EH, MIC	12.5	25	25	25	25			25	25	6.25
MBC	12.5	25	50	50	50	25		50	50	50
CMB/CMI	1	1	2	2	2	50		2	2	8
						2				
EM1, CMI	50	25	25	25	50			50	50	50
CMB	>50	50	50	50	>50	50		50	50	>50
CMB/CMI	-	2	2	2	-	>50		1	1	
				-						
EM2, CMI	>50	50	50	50	50			>50	>50	>50
CMB	>50	50	50	50	50	50	-	>50	>50	>50
CMB/CMI		1	1	1	1	50				
						1				
EC, CMI	50	50	>50	>50	>50			>50	>50	>50
CMB	50	50	>50	>50	>50	>50		>50	>50	>50
CMB/CMI	1	1				>50				
EA,CMI	50	50	50	50	>50			>50	>50	>50
CMB	>50	50	50	50	>50	>50		>50	>50	>50
CMB/CMI		1	1	1		>50		-	-	-
Gentamycine 20 mg/ml	S	S	S	S	S			S	S	S

C. a; S. a: *Staphylococcus aureus*; E. c: *Escherichia coli*; P. a: *Pseudomonas aeruginosa*; K. p: *Klebsiella pneumoniae*; C. d: *Citrobacter diversus*; EH: hydroalcoolic Extract; EM1: non delipided methanolic extract; EM2: delipided methanolic extract; EC: chloroformic extract; EA: aqueuse extract; S: Sensitive; - : non given antibiotic capacity.

made it possible to determine the Minimum Inhibitory Concentrations (MIC), the Minimum Bactericidal Concentrations (MBC) and the antibiotic potency (MBC/MIC) of each extract. The antibiotic potency was considered bactericidal if MBC / MIC <1; it was bacteriostatic if 1 <MBC / MIC. The results are shown in Table 3. The analysis results showed that the hydroalcoholic extract was the most active on all of the microorganisms studied while the chloroform and aqueous extracts were the least active. The hydroalcoholic extract exerts a bactericidal effect on Candida albicans with MIC = 12.5 mg/ml and on Citrobacter diversus with MIC = 6.25 mg / ml. The effect on the other microbial strains tested was bacteriostatic. The extract EM1 inhibited the growth of all the germs tested. There was a bactericidal effect at 50 mg / ml on salmonella OMB and *Klebsiella pneumonia* but bacteriostatic effect was observed on *S. aureus*. The EM2 extract inhibited *S. aureus* and E. coli growth at 50 mg/ml. Similarly, at 50 mg/ml EC and EA extract inhibited the growth of *S. aureus* and *C. albicans*. All strains tested were sensitive to gentamycin, the reference antibiotic (20 mg / ml) was used as control. The results obtained therefore indicate that the antimicrobial activity of *M. scaber* extracts could be attributed to phenolic and alkaloids compounds plant extract. However, the aqueous extract richest in phenolic compounds was the

Table 4. Viability of microorganisms as a function of time.

Number of colony/strains \ Time	0 min	15 min	30 min	45 min	24 h	48 h
E. coli	108	460±40	9±1	1±1	-	-
E. coli ATCC 25922	108	0±0	0±0	0±0	-	-
S. aureus	108	108	0±0	0±0	-	-
S. aureus ATCC 25923	108	108	0±0	0±0	-	-
S. aureus ATCC 29213	108	108	108	27±2	-	-
P. aeruginosa ATCC 27853	108	108	3±1	1±1	-	-
Salmonella OMB	108	1973±428	9±6	2±1	-	-
K. pneumoniae	108	57±1	1±1	0±0	1112±171	1310±105
C. diversus	108	1600±401	1380±80	1287±173	-	-
C. albicans	108	108	24±6	2±1	-	-

least active on the microbial strains tested while the hydroalcoholic extract less concentrated in these compounds was the most active on all the microorganisms studied.

Gbaguidi et al. (2005) showed that the alkaloid Azaanthraquinone benzo (g) isoquinoline isolated from an alcohol extract of M. scaber was responsible for this antibacterial activity. MIC was 19 µg/ml on S. aureus ATCC 25923, 150 µg/ml on E. coli ATCC 25922 and >10 mg / ml on P. aeruginosa ATCC 27853. Alkaloids were organic substances with complex alkaline molecular structures with high pharmacological activity at low concentration (Bruneton, 1999). Among these pharmacological properties were the antimicrobial properties (Karou et al., 2006).

On the other hand, the antimicrobial activity of seven polyphenolic compounds isolated from methanol extract was examined by Bisignano et al. (2000). They showed that gallic acid and 3, 4, 5-trimethoxybenzoic acid isolated from the said plant inhibited the standard and clinical strains of S aureus (MIC 3.90 and 0.97 µg/ml), and that 4-methoxyacetophenone and 3, 4, 5-trimethoxyacetophenone also inhibited the reference and clinical strains of C. albicans (MIC 1.95 and 0.97 µg / ml). Thus, the antimicrobial activity of M. scaber was due to the synergistic action of the phenols and alkaloids of this plant.

In addition, Bisignano et al. (2000) also tested total methanol extracts and found MICs of 31, 25 µg / ml on S. aureus ATCC 25923 and 62.50 µg / ml on C. albicans. Drying and storage of the extracts would influence the quality of the extracts. The fresh plant of M scaber contains harounoside, a molecule with antimicrobial activity whereas it was absent in the dry plant (Harouna et al., 1995).

Similarly, Karou et al. (2015) showed that the conservation of extracts had a negative impact on their pharmacological quality. Indeed, the author has demonstrated that the phenols in contact with oxygen undergo an auto-oxidation and give insoluble polymers of high molecular weights that penetrate the bacterial wall. To better understand the action of this plant, the hydroalcoholic extract was selected for its interesting activity to conduct a kinetic on the microbial strains studied. The results of this test are recorded in Table 4. The hydroalcoholic extract of *M. scaber* exerted a bactericidal effect at 100 mg/ml on all of germs tested. After 45 min of contact, growth of *E. coli* ATCC 25922, *S. aureus* 25922, K. pneumoniae was completely inhibited. This bactericidal action was observed from the 15th min for *E. coli* strain, 30th min for *C. albicans* and 45th min for S. aureus ATCC 29213. For *Citrobacter diversus* strain, there was an escape phenomenon. Indeed, the effect exerted by the extract on this strain would be of bacteriostatic type 15 min after contacting the germs.

Conclusion

M. scaber was an herbaceous plant well known to populations of several African countries. Renowned for its antifungal and antimicrobial activities, its aerial parts are mostly used for the treatment of dermatoses and mycoses by indigenous populations. These popular practices have attracted the attention of researchers who had carried out several studies in different research laboratories to confirm the traditional uses and understand the active principles responsible for the activities of the plant. Thus, in vitro tests of the antifungal and anti-microbial activity of M. scaber extracts carried out on several germs had proved to be active and thus confirmed the traditional practices. In addition, the molecules responsible for its activities have been isolated from extracts and even from the essential oil of the leaves of the plant. The results obtained can benefit some pharmaceutical companies who make ointments and cosmetic soaps. From all this work, M. scaber has an international reputation and, as a result, work on this

rubiaceae must continue on other aspects, especially ash analysis, other insect-destroying agricultural products and the ability of the plant to potentiate the action of the anti-microbial agents that certain bacteria defy etc. in order to optimize its use for the benefit the indigenous populations.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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