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Mangifera indica Bark Essential Oil: Chemical Composition and Biological Activities in Comparison with Aqueous and Ethanol Extracts

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Ethnobotanic surveys have revealed the use of *Mangifera indica* L. (Anacardiaceae) bark for the treatment of infectious diarrhea. The essential oil of *M. indica* bark is described for the first time for its chemical composition, radical scavenging activity (DPPH method) and antimicrobial properties. The total phenols content of its water and ethanol bark extracts as well as their radical scavenging and antimicrobial properties were also evaluated. Four commercial plant extracts were also studied for a comparison purpose. The antimicrobial activities were measured for all samples against three Gram (-): *Escherichia coli*, *Salmonella enteritidis*, *Shigella* and two Gram (+): *Staphylococcus aureus* and *Bacillus cereus* bacteria. The *M. indica* bark essential oil was characterized by the association of two major sesquiterpenes: (*E*)- β -caryophyllene (60.3%) and α -humulene (36.7%). It presented the lowest ratio of concentration to inhibition zone diameter on all the strains. The aqueous and ethanol extracts of *M. indica* bark were characterized by high contents of total phenols compounds and high radical scavenging activity compared to the essential oil. Finally, the interesting combination of the antibacterial and antiradical activities of the aqueous *M. indica* bark extract justifies the traditional use of this plant part in decoction form for the treatment of diarrheal infections.

Keywords: *Mangifera indica*, Bark, Essential oil, Aqueous extract, Ethanol extract, Antimicrobial activity, Radical scavenging activity.

Mangifera indica L. (*M. indica*) is a tree of the Anacardiaceae family native in the east of India (Himalaya Mountains); it is now cultivated like fruit tree in all the tropical countries [1]. It has been used in the Ayurvedic and indigenous medical systems for over 4000 years [2]. All the parts of the plant are employed in traditional medicine and some of its medicinal relevance includes treatment of diarrhea and dysentery [3]. Infectious diarrhea is a significant and crucial world problem in Africa, leading in general to a sickness period between 5 to 7 days for adults, while it causes 0.2% death among children of less than 5 years [4].

The role of bacteria has been reported in most cases of fatal diarrhea [5] hence needing the use of anti-infective agents in their management. More than 80% of people in rural African communities still rely on indigenous medicine as a primary source of health care; in this context, plants and their essential oils or extracts are potentially useful sources of antimicrobial compounds [6]. During a bacterial infection, uncontrolled overproduction of reactive oxygen species (ROS) and nitric oxide (NO) is observed resulting in oxidative damage to cells and tissues. The role of these reactive metabolites in inflammatory disease was reported by Pavlick *et al.* [7]. Hence, essential oils or plant extracts with the two effects, antibacterial and antiradical, should be suitable in the treatment of infectious diarrhea. In this regard, some studies have reported on the biological properties of *M. indica* bark [2,8] and on the role of mangiferin, identified in its aqueous bark extract [8] as well as in the leaves [9]. Moreover, several studies reported the antibacterial [8, 10-12] and antioxidant [12-14] activities of *M. indica* bark extracts obtained with different solvents. Nevertheless, we did not find any information on the chemical composition or the antibacterial and antioxidant activities of its essential oil. Therefore, the aims of this study were to (1) assess the *in vitro* antimicrobial activity of essential oil in comparison with aqueous and ethanol extracts from *M. indica* bark, (2) determine the

Table 1: Chemical composition of *M. indica* bark essential oil collected in Cameroon.

N°	Compounds	LRI ^a	LRI ^b	%	Identification methods
Monoterpenes					
1	1,8-Cineole	1035	1215	<0.1	LRI,MS
Sesquiterpenes					
2	β -Elemene	1364	1609	<0.1	LRI,MS
3	(<i>E</i>)- β -Caryophyllene	1435	1637	60.3	GC,LRI,MS
4	α -Humulene	1468	1702	36.7	GC,LRI,MS
5	α -Curcumene	1487	1769	0.2	LRI,MS
6	β -Selinene	1489	1725	<0.1	LRI,MS
7	α -Selinene	1494	1728	0.2	LRI,MS
8	Caryolan-8-ol	1572	2046	0.9	LRI,MS
9	Caryophyllene oxide	1584	1979	0.5	LRI,MS
10	Humulene epoxide II	1609	2008	0.5	LRI,MS
Total identified compounds				99.3	

N°: elution order on apolar column (HP-5); ^a Linear Retention Index on apolar (HP-5) column; ^b Linear Retention Index on polar (DB-Wax) column; Identification methods: GC, identification based on co-injection with authentic sample; LRI, MS: identification based on comparison of linear retention index and mass spectrum with literature data; %: relative area percentage.

chemical composition of its essential oil, (3) estimate the total phenols content of the aqueous and ethanol extracts and (4) evaluate their radical scavenging activity using the DPPH (2,2-diphenyl-1-picrylhydrazyl) test.

The essential oil and extracts from *M. indica* bark were obtained with the following yields (w/w): 0.04% for the essential oil, 7.4% for the aqueous extract and 1.8% for the ethanol extract. GC and GC-MS analyses of the essential oil allowed the identification of ten components amounting to 99.3% of the total chemical composition (Table 1); the oil was characterized by two major sesquiterpenic compounds: (*E*)- β -caryophyllene (60.3%) and α -humulene (36.7%). *M. indica* bark essential oil is described for the first time. Analysis of essential oils obtained from *M. indica* leaves collected in Nigeria [15] or Brazil [16] showed chemical compositions dominated by α -gurjunene (23.6-24%) and β -selinene (20.6-24%). β -caryophyllene represented only 11.2% and α -humulene 10.8-7.2% of these samples.

Table 2: Sensitivity of bacteria to the extracts and essential oil from *M. indica* bark.

Extracts	Sample concentrations (ppm)				
	(Inhibition zone (IZ) diameter \pm sd (mm))				
	Gram -		Gram+		
	<i>E. coli</i>	<i>S. enteritidis</i>	<i>Shigella</i>	<i>S. aureus</i>	<i>B. cereus</i>
Essential oil	300 (33.0 \pm 1.4)	300 (43.0 \pm 0.1)	300 (39.8 \pm 0.4)	300 (29.0 \pm 0.1)	300 (29.0 \pm 0.1)
Aqueous extract	1200 (9.7 \pm 0.6)	1200 (27.7 \pm 1.5)	1200 (28.5 \pm 2.1)	1200 (31.0 \pm 2.8)	1200 (20.7 \pm 0.3)
Ethanol extract	300 (10.7 \pm 0.6)	1200 (10.5 \pm 1.3)	NI	1200 (10.3 \pm 1.5)	1200 (11.7 \pm 1.2)

E. coli=*Escherichia coli*, *S. enteritidis*=*Salmonella enteritidis*, *S. aureus*=*Staphylococcus aureus*, *B. cereus*=*Bacillus cereus*, NI= No Inhibition observed at 1200 ppm, Values represent average of triplicates \pm standard deviation.

The antimicrobial activities of *M. indica* bark extracts and essential oil on the most common strains implicated in infectious diarrhea such as *Escherichia coli*, *Salmonella enteritidis*, *Shigella*, *Staphylococcus aureus* and *Bacillus cereus* were assessed by a disc diffusion method through the inhibition zone (IZ) diameter measurement and by a macrodilution method determining the MIC and MBC values [17]. The IZ diameters (expressed in mm) are presented in Table 2. The highest IZ diameters (29-43 mm) were observed with the essential oil at 300 ppm for all the strains, the three more sensitive strains being Gram negative bacteria. Globally, the aqueous and ethanol extracts were less active than the essential oil: the IZ diameter obtained with the ethanol extract at 300 ppm was only 10.7 mm against *E. coli* and a significant inhibition was only observed at 1200 ppm in the other cases.

The antimicrobial activities of the essential oil and extracts obtained from *M. indica* bark were also evaluated using *in vitro* broth dilution method. The corresponding antibacterial activities are presented in Table 3 along with those of four commercial samples selected for a comparison purpose: clove and rosemary essential oils as well as rosemary supercritical CO₂ extract and green tea ethanol extract.

Considering the three essential oils, the most active was the one obtained from the *M. indica* bark. The best inhibitory effect was observed against *S. aureus* (MIC=9.37 ppm) and *E. coli* (MIC=75 ppm). It was less active on *Shigella* and *B. cereus* (MIC=150 and 300 ppm respectively) while it was inefficient on *S. enteritidis* growth (MIC >1200). A bactericidal effect (MBC/MIC=2) could be observed with *E. coli*, *Shigella* and *B. cereus*. These results may be in grand part ascribed to β -caryophyllene (60.3%), for which antimicrobial activity has already been demonstrated (MIC 1.84 ppm, 0.6 ppm and 1.84 ppm on *B. cereus*, *S. aureus* or *E. coli* respectively) [18]. The higher than expected MIC values obtained with the *M. indica* oil might indicate an antagonist effect of some of the other components or the strain specificity. On the other hand, the rosemary essential oil was inefficient at 1200 ppm on most of the tested strains while the clove essential oil presented an inhibitory effect on *E. coli* at 75 ppm and a bactericidal action at 1200 ppm on *S. enteritidis* and *S. aureus*. This antimicrobial action could be associated to a combinatory effect of eugenol (85.7%) and β -caryophyllene (11.6%).

Regarding the *M. indica* bark extracts, they were globally less efficient than the essential oil with the exception of their action on *S. enteritidis*, a microorganism widely implicated in diarrhea. The bactericidal effect of *M. indica* ethanol extract observed at 37.5 ppm on *S. aureus* and at 600 ppm on *B. cereus* should be noted. Rosemary supercritical extract was inefficient on all the strains while the green tea ethanol extract presented a significant inhibitory effect on *E. coli* at 18.75 ppm.

The phenols and reductants content of the *M. indica* bark extracts were obtained using the Folin-Ciocalteu (FC) colorimetric method [19] from the equation $A_{735\text{ nm}} = 0.0106c$ ($r^2 = 0.995$) in which $A_{735\text{ nm}}$ represents the absorbance at 735 nm and c the gallic acid concentration, in mg/L; the phenols contents were expressed as gallic acid equivalent (GAE) per gram of extract as follows in Table 4. The results were compared with those obtained with the two commercial extracts: rosemary supercritical CO₂ extract and green tea ethanol extract. The essential oils were not subjected to this titration due to experimental constraint. As observed in Table 4, the results indicate a more efficient extraction of the phenols and reductants compounds from *M. indica* bark by aqueous treatment. The two aqueous and ethanol *M. indica* bark extracts were highly rich in phenolic compounds (696 and 600 mg GAE/g extract, respectively); these values are close to that of the green tea ethanol extract, which is known for its high content of catechins [20]; the *M. indica* bark extracts were even richer than the rosemary supercritical CO₂ extract, which is used industrially as a source of natural antioxidant.

The radical scavenging capacities of all samples, expressed as gallic acid equivalent (GAE) per gram of extract (Table 4), were determined by the DPPH method from the equation $SC\% = 27.86c$ ($r^2 = 0.995$) in which $SC\%$ represents the radical scavenging percentage and c the gallic acid concentration in mg/L. A relationship between the phenols and reductants content and the radical scavenging activity was clearly observed. Examination of Table 4 indicates that the essential oils were generally less active than the extracts, excepted the case of the clove oil owing to its high eugenol content. The green tea ethanol extract presented the best radical scavenging activity while the *M. indica* bark aqueous extract was more efficient than the ethanol extract.

In conclusion, this study reports for the first time on the *M. indica* bark essential oil in terms of its chemical composition as well as its antibacterial and radical scavenging activities. This essential oil presented the best antibacterial activity while the aqueous extract seems to be the most interesting, because it combines good antibacterial and antiradical activities, which justifies the traditional use of *M. indica* bark in decoction or infusion form as plant based diarrhea medication. Finally, our results suggest that the essential oil and the aqueous extracts could be combined at low concentrations for a most efficient treatment.

Table 3: Antibacterial activities of the solvent extracts and essential oil from *M. indica* bark and of four commercial plant extracts.

Tested bacteria	<i>E. coli</i>			<i>S. enteritidis</i>			<i>Shigella</i>			<i>S. aureus</i>			<i>B. cereus</i>		
	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
<i>M. indica</i> essential oil	75	150	2	>1200	>1200	ND	150	300	2	9.37	300	32	300	600	2
rosemary essential oil	600	>1200	ND	>1200	ND	ND	>1200	ND	ND	>1200	ND	ND	>1200	ND	ND
clove essential oil	75	300	4	1200	1200	1	600	>1200	ND	600	1200	2	300	>1200	ND
<i>M. indica</i> aqueous extract	18.75	>1200	ND	150	1200	8	>1200	>1200	ND	75	600	8	150	1200	8
<i>M. indica</i> ethanol extract	150	1200	8	600	1200	2	150	1200	8	37.5	37.5	1	600	1200	2
rosemary supercritical CO ₂ extract	>1200	ND	ND	>1200	ND	ND	1200	>1200	ND	>1200	ND	ND	>1200	ND	ND
green tea ethanol extract	18.75	1200	64	>1200	ND	ND	300	1200	4	300	1200	4	300	>1200	ND

E. coli=*Escherichia coli*, *S. enteritidis*=*Salmonella enteritidis*, *S. aureus*=*Staphylococcus aureus*, *B. cereus*=*Bacillus cereus*, NI= No Inhibition observed at 1200 ppm

Table 4: Phenols and reductants content (Folin-Denis method) and radical scavenging activity (DPPH test) relative to gallic acid.

	Folin-Denis (mg GAE/g extract)	DPPH (mg GAE/g extract)
Essential oils		
<i>M. indica</i>	/	1.5±0.6
Rosemary	/	0.07±0.00
Clove	/	535±9
<i>M. indica</i> aqueous extract	696±4	317±1
<i>M. indica</i> ethanol extract	600±5	219±7
green tea ethanol extract	956±8	466±0
rosemary supercritical CO ₂ extract	153±3	43±1

GAE=Gallic acid equivalent; DPPH= 2,2-diphenyl-1-picrylhydrazyl; / = not done. Values represent average of triplicates ± standard deviation.

Experimental

Chemicals: All solvents (ethanol, methanol and DMSO) were purchased from Sigma–Aldrich. 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,3,5-triphenyl tetrazolium chloride (TTC), gallic acid and Na₂CO₃ were acquired from Sigma–Aldrich. Water was purified using the Mili-Q-system (Milipore). Folin-Denis' reagent was purchased from Fluka Analytical (France).

Commercial plant extracts:

- Essential oil of rosemary 1,8-cineole chemotype (*Rosmarinus officinalis* L.) leaves n°NHE0273-7/815 (eucalyptol 51.7%, α -pinene 11.5%, β -pinene 9.3% and camphor 8.7%) purchased from Aroma Zone (France).
- Essential oil of clove (*Syzygium aromaticum* L.) buds (eugenol 85.7% and (*E*)- β -caryophyllene 11.6%) purchased from Sentaromatique (France).
- A supercritical CO₂ extract from rosemary (*Rosmarinus officinalis* L.) leaves n°OCO20024-4/1241 (diterpenic phenols 13-15% of which carnosic acid 9%) purchased from Aroma Zone (France).
- An ethanol extract from green tea leaves (*Camellia sinensis* L.) n°203122 (catechins [20]) purchased from Payan Bertrand (France).

Plant material and extraction procedure: *M. indica* bark was collected at the University of Yaoundé I (Cameroon) in July 2015. The botanical identification and authentication were carried out at the National Herbarium of Cameroon (Yaoundé) where voucher specimen is kept: 18646/SRF Cam. Fresh bark was used to get essential oil while the aqueous and ethanol extractions were performed on bark samples after drying at 30°C under a shell.

Batch of 1000 g of fresh bark was chopped into small pieces and essential oil was obtained by hydrodistillation for 6-8 h using a Clevenger-type apparatus. After separation by decantation, the oil was dried over anhydrous sodium sulfate and, after filtration, stored in sealed flasks at 4°C until tested and analyzed. The aqueous and ethanol extractions were carried out by macerating the dried powdered bark (four batches of 250 g of plant sample in 2 L of water or ethanol 96% at room temperature) for 24 h with frequent stirring every 2 h. After filtration on Whatman N°3 paper, the filtrates of each extract were gathered and lyophilized or concentrated by evaporation at 65°C, to give the aqueous and ethanol extracts respectively. The yields (w/w) were calculated according to the weight of plant material.

Antimicrobial activities: The antibacterial activity of the essential oils and extracts were individually tested against three Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Salmonella enteritidis* 155A, *Shigella*) and two Gram-positive bacteria (*Staphylococcus aureus* NCTC 10652, *Bacillus cereus* ATCC 11966). *Shigella* was a clinical isolate obtained from the University Hospital Center of Yaoundé (Cameroon), the others were kindly offered by the Laboratory of Food Microbiology, University of Bologna (Italy). Strains stored at -80°C were cultured at 37°C for 24 h twice in Brain Heart Infusion (BHI) broth before being used.

Disc diffusion method: This method was carried out in accordance with CLSI recommendations [17]. Samples were dissolved in 10% DMSO then diluted to 5 final concentrations of 1200, 600, 300, 150 and 75 ppm. Briefly, 200 μ L microbial culture of each bacteria species (10⁶ cells/mL) were inoculated on a solidified Broth Heart Infusion (BHI) in a Petri dish; then 6 mm diameter whatman paper discs soaked with 30 μ L of the diluted samples were deposited on the inoculated surface of the BHI plates. Discs soaked with DMSO were used as negative control. The Petri dishes were then incubated at 37°C for 24 h. The growth inhibition zone diameter (IZ, mm) was measured to the nearest mm. Each experiment was performed in triplicate and the results presented in terms of the concentration that produced the highest inhibition diameter.

Serial broth macrodilution method: The method was carried out in accordance with CLSI recommendations [17]. A stock solution was first prepared by diluting the respective sample in 10% DMSO. Simultaneously, 10⁵ cells/mL of bacteria inoculum was prepared in Mueller Hinton broth from an overnight broth culture. Subsequently, 40 μ L of the stock solution was added to 4 mL of bacteria inoculum to reach 1200 ppm as first test concentration. Then, from this concentration, we proceeded to twofold dilution using bacteria inoculum to obtain concentrations ranging from 1200 ppm to 9.37 ppm followed by incubation at 37°C for 24 h (after mixing with a vortex). Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were defined as in [17]. The presence of viable bacterial after incubation was assessed by adding 200 μ L of a 10 ppm solution of TTC; color change to red indicated the presence of viable cells. In the case of *E. coli*, the appreciation of growth was done by evaluating the presence of turbidity. The antibacterial effect was deemed bactericidal or bacteriostatic depending on the ratio: MBC/MIC. Indeed, if MBC/MIC=1-2, the effect is bactericidal and if MBC/MIC=4-16, the effect is bacteriostatic [21].

Determination of phenols and reductants content: phenols content was estimated by the Folin-Ciocalteu (FC) colorimetric method [19] with some experimental adaptation. Gallic acid (GA) was used as calibration standard. Briefly, 0.5 mL of gallic acid solutions (c=10-50 mg/L) was mixed with 0.25 mL FC reagent (2N). After five minutes, 1.25 mL sodium carbonate aqueous solution (20% w/v) was added. The mixture was shaken and left during 1 h at room temperature. Absorbance was measured at 735 nm at different concentrations (c) of GA and the linear regression A=f(c) carried out with Microsoft excel. The same protocol was used with the samples at two concentrations in order to evaluate their phenols content, expressed in mg GA (GAE) per gram sample.

Radical scavenging capacity: The antioxidant activity was assessed as described by Nyegue *et al.* [22] using a 100 μ M ethanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). 100 μ L of a methanol solution of the antioxidant at different concentrations were added to 1900 μ L of the DPPH solution. The control, without antioxidant, was represented by the DPPH solution containing 100 μ L of methanol. Absorbance was measured at 517 nm after 120 min and the scavenging percentage of DPPH was obtained from the following equation $SC\% = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$. SC% were evaluated at different concentrations of GA (0.5-2.5 mg/L) to get a linear calibration curve (SC% values 0-60). The samples were evaluated at two concentrations, selected in order to obtain SC% values comprised in this linear part of the calibration curve; the radical scavenging capacities were expressed in mg of GA equivalent (GAE) per gram of sample. All the spectrophotometric measures were performed with a SAFAS UV mc² spectrophotometer, equipped with a thermostated cells-case at 30°C.

Gas chromatography analyses: GC analyses were performed on a Thermo Scientific gas chromatograph, model TRACE 1300, equipped with a flame ionization detector (FID) using a TG-5MS (5% phenyl methylpolysiloxane) capillary column (30 m x 0.25 mm, film thickness 0.25 μ m) and a TG-WAXMS (polyethylene glycol) capillary column (30 m x 0.25 mm, film thickness 0.25 μ m); N₂ was the carrier gas at 0.5 mL/min; injection of 2 μ L of a 10/100 CH₂Cl₂ solution (split ratio 1:20); injector temperature 220°C, detector temperature 250°C; oven temperature program 60-220°C at 3°C/min then kept at 220°C during 17 min. The linear retention indices (LRI) of the components were calculated with reference to a series of *n*-alkanes. The percentage composition of the essential oil was computed by the normalization method from the GC-FID peak areas on the DB-5 capillary column, response factors being taken as one for all compounds.

Gas chromatography-mass spectrometry: GC-MS analyses were performed using an Agilent 5977 apparatus MSD series, equipped with two silica capillary columns: HP-5 MS (5% phenyl methyl polysiloxane; 30 m x 0.25 mm; film thickness 0.25 μ m) and HP-INNOWAX (30 m x 0.25 mm; film thickness 0.25 μ m) interfaced

with a quadrupole mass detector; source temperature 230°C, quadrupole temperature 150°C; oven temperature program: 60°C for 2 min, 60-240°C at 3°C/min, then kept at 240°C during 8 min; injector temperature, 240°C; MS transfer line temperature, 250°C; carrier gas, helium at a flow rate of 0.7 mL/min; injection of 1 μ L of a 10/100 CH₂Cl₂ solution (split ratio 1:20); ionization voltage, 70 eV; scan range 33-400 amu; scan rate, 1.56 scan/s. The identification of the constituents was based on comparison of their relative retention indices with either those of authentic samples or with published data in the literature [23] and by matching their mass spectra with those obtained with authentic samples and/or the NIST98 and FFNSC 2.L. libraries spectra.

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