

Phytochemical screening and study of comparative antibacterial activity of aqueous and alcoholic extracts of the leaves and barks of *Terminalia catappa* on multiresistant strains.

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ABSTRACT

Objectives: In this study, methanol, ethanol and aqueous extracts of *Terminalia catappa* L. leaves and barks were compared for antibacterial activity against clinical (sensitive and multidrug-resistant) and ATCC strains of *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* (MRSA).

Methodology and Results: The confirmation of the *Enterobacteriaceae* Extended-Spectrum- β -Lactamases (ESBL) producing strains was done by the double-disc synergy tests, the MRSA by disk diffusion method (cefoxitin 30 μ g) according to the methods of CA-SFM, 2009. The agar dilution and broth dilution method was used for the determination of the antimicrobial parameters (MIC and MBC) on these sensitive, ESBL producing and MRSA strains. The bark extracts generally showed better activity compared to leaves extracts; but methanolic extracts of barks were the most active of all the extracts, exhibiting a broad-spectrum activity. However, *Staphylococcus aureus* were sensitive compared to *Escherichia coli* and *Klebsiella pneumoniae*. Antibacterial activity showed value ranged from 0.52 ± 0.13 to 4.16 ± 1.04 mg/ml (MIC) and 1.56 to 10.42 ± 2.08 mg/ml (MBC). Phytochemical screening showed the presence of Flavonoids, Alkaloid, Steroid, Terpenoids, Gallic Tannins, Saponins and with the exception of the aqueous extract for Coumarins.

Conclusions: The results obtained suggest that methanolic extracts of *T. catappa* barks can be used in treating bacterial diseases.

Key words: Phytochemical Screening; Comparative Antibacterial activity; *Terminalia catappa*; multiresistant strains.

INTRODUCTION

Progress of antibiotic therapy seemed to have solved almost all common problems in infectious diseases. Subsequently, the dramatic emergence of resistance in bacteria previously susceptible to multidrugs becomes a serious problem (Ndiaye, 2005). For over 20 years, there has been a steady increase in antibiotic resistance in hospitals and

the community. This is a real public health problem and thus puts into question the validity of the antibiotics currently available. (WHO, 2002a, Stahl, 2006). In Côte d'Ivoire and worldwide, *Staphylococcus aureus* resistant to methicillin (MRSA) and *Enterobacteriaceae* Extended-Spectrum- β -Lactamases (ESBL) producing

Escherichia coli and *Klebsiella pneumoniae* are among the most common resistant organisms (Rong-Dih et al., 2005; Sorlózano et al., 2007; Guessennd et al., 2008; Belmonte et al., 2010; Gandolfi et al., 2011; Kacou-N'Douba et al., 2011). The lack of new molecules, as well as the diversity of resistance mechanisms require the development of new therapeutic agents in the fight against this scourge (Adebayo-Tayo and Adegoke, 2008; Nyenje et al., 2011). Medicinal plants are an important source of new drugs adapted to the financial resources and the socio-cultural populations and especially since, they have fewer side effects (Maghrani et al., 2005; Doughari et al., 2007). According to WHO estimates (2002 b), more than 80% of the population in Africa still use traditional medicine to satisfy their health needs.

MATERIALS AND METHODS

Plant material: The plant material consisted of bark and leaves of *Terminalia catappa* collected on the campus of the University of Cocody between June-July 2009. They were authenticated at the National Centre of Floristic the University of Cocody. The leaves and bark of *Terminalia catappa* were washed, cut and dried in sunlight at room temperature (25 ° to 30 °C) for about two weeks to three weeks. Finally, dry leaves and barks were pulverized using a grinder to obtain a fine powder, which was used for the preparation of total extracts aqueous ethanol and methanol.

Bacterial strains: The isolates included reference bacterial strains: (*E. coli* ATCC 25922, *S. aureus* ATCC 25923), clinical sensitive strains (*S. aureus* 079y/09, *E. coli* 766c/09 and *K. pneumoniae* 768c/09) and resistant strains (*S. aureus* 762c/08, *S. aureus* 908c/09, *E. coli* 066/08, *E. coli* 555/08, *K. pneumoniae* 485/08, *K. pneumoniae* 773c/09 and one beta-lactam antibiotic). These were provided by the collection of Laboratory of Virology & Bacteriology of Institute Pasteur of Abidjan in Ivory Coast.

Preparation of plant extracts

Preparation of the aqueous extract total: This extraction was performed according to the method described by Ackah et al. (2008). Hundred grams (100 g) powder plant were stirred vigorously in 2 liters of distilled water using an electric mixer. The mixture was filtered twice through cotton wool, then through Whatman filter paper (N°1). The filtrate was evaporated to dryness using under vacuum in a rotary evaporator (Buchi) at 60°C to obtain a powder.

Preparation of alcoholic extracts: Preparing ethanolic and methanolic extracts was made using the same method described above using the respective

Among the most utilized plants is *Terminalia catappa* (Badamier, Cocoma) (Fofana, 2004), traditionally used for its antiparasitic, anti-infectious, anti diarrheal, anti hypertensive and anti diabetic property. It is also used in the treatment of asthma, skin diseases, gastrointestinal diseases, respiratory, cardiovascular and rheumatic diseases, scabies, leprosy, prurigo, sores, headache disorders, gonorrhoea and leucorrhoea (Ackah et al., 2008; Sumitra and Nair, 2008, Annegowda et al., 2010;) Given the properties of this plant, our team wanted to study the validity of its antimicrobial qualities. Thus, we chose to test the antibacterial activity against ESBL producing and MRSA by extracts of bark and leaves of *Terminalia catappa*

solvents ethanol and methanol. The filtrate is concentrated on a rotary evaporator under reduced pressure at 50°C and then dried in an oven at 40°C. Extracts obtained were stored in a refrigerator until used for bioassays.

Extract yield: The yield was calculated according to the formula:

$$R (\%) = M_1 \times 100 / M_0$$

R (%): Extract yield; M₁: extract quantity (g); M₀: powder quantity of leaves or barks of *T. catappa* used by extraction (g). (Bssaibis, 2009)

Phytochemical analysis: The freshly prepared extracts were subjected to standard phytochemical analyses to test for the presence of the phytoconstituents Alkaloids, Saponins, Tannins, terpénoïdes, flavonoid, coumarin and substances quinoniques (Adebayo-Tayo et Adegoke, 2008, Babayi, 2004).

Confirmatory test for ESBL-producing *K. pneumoniae* and *E. coli* isolates: Antimicrobial susceptibility of Gram-negative clinical strains was determined by standard antibiogramme, according to the manufacturer's recommendation (CASFM, 2009).

Confirmatory test for MRSA: Susceptibility to methicillin was determined by the method of Kirby-Bauer diffusion according to recommendations of the susceptibility of the French Society of Microbiology (CASFM, 2009). The antibiotic was tested cefoxitin (30 µg). The quality control was performed with reference strains *E. coli* ATCC 25922 and *S. aureus* ATCC 25925.

Paper disc agar diffusion method: Antibacterial activity of the aqueous and organic extracts of

Terminalia catappa leaves and bark extracts sample was evaluated by the paper disc diffusion method described by Doughari et al., (2007); Adebayo-Tayo and Adegoke (2008). Mueller-Hinton medium (GMH) was used for culture of bacteria. For determination of antibacterial activity, bacterial cultures were adjusted to 0.5 McFarland turbidity standard and inoculated onto GMH plates. Sterile filter paper discs (diameter 6 mm) impregnated with 10µl of extract dilutions was reconstituted in minimum amount of solvent at concentration of 100 mg/ml, and applied over each of the culture plates previously seeded with the 0.5 McFarland. Bacterial cultures were then incubated at 37°C for 24h. All the results were compared with the disc of standard antibacterial antibiotic ceftazidim (30µg) and cefoxitin (30 µg) for Enterobacteriaceae (*Escherichia coli* and *Klebsiella pneumoniae*) and *Staphylococcus aureus* respectively. The antibacterial activity was evaluated by measuring the diameter of inhibition zone. The experiment was repeated thrice and the mean of the diameter of the inhibition zones was calculated.

Determination of minimal inhibitory concentration and minimal bactericidal concentration: The Minimal Inhibitory Concentration (MIC) and the Minimal Bactericidal Concentration (MBC) was determined according to Wilkinson and Gentry revised by

N'guessan et al. 2007. MIC of the extracts of *Terminalia catappa* leaves and bark was estimated for each of the test organisms in triplicates. 1.8 ml of Mueller-Hinton broth (BMH) was added into 0.2 ml of varying concentrations of the extracts (100.0; 50.0; 25.0; 12.50; 6.25; 3.125; 1.56; 0.78; 0.39 and 0.0195 mg/ml). Then, a loopful of the test organism previously diluted to 0.5 McFarland turbidity standard was introduced to the tubes. A tube containing nutrient broth only was seeded with the test organisms as described above to serve as control. Tubes containing bacterial cultures were then incubated at 37°C for 24 h. After incubation, the tubes were then examined for microbial growth by observing for turbidity. To determine MBC, a loopful of broth was collected from tubes, which did not show any growth and inoculated on sterile Mueller-Hinton agar (GMH) by streaking. Nutrient agar (GMH) only were streaked with the test organisms respectively to serve as control. Plates inoculated with bacteria were then incubated at 37°C for 24 hours. After incubation, the concentration at which no visible growth was seen was noted as the minimum bactericidal concentration.

Statistical analysis: Experiments were conducted in triplicate. Data were analyzed by one-way ANOVA followed by Dennett's t-test using InStat® (Graph Pad software, U.S.A). At 95% confidence interval $p < 0.05$ was considered statistically significant.

RESULTS

Extract efficiency: The total mass of plant material extracted by the different solvents (water, ethanol and methanol) from 100 g of plant material is presented in the Figure 1. Yields for the various extracts showed values between 11.87 ± 0.13 % and 19.75 ± 0.17 %. Among the three solvents used, methanol gave the highest yields. On the organ used, the highest yields were obtained with bark.

Phytochemical analysis: The result of the phytochemical analysis show that the Methanolic, Ethanolic and Aqueous of leaf and bark extracts of *T. catappa* are rich in Flavonoids, Alkaloid, Steroid and Terpenoids, gallic Tannins, Saponins and Coumarins with the exception of the aqueous extract (Figure 2). The presence of these constituents has been reported to account for the exertion of antimicrobial activity by plant.

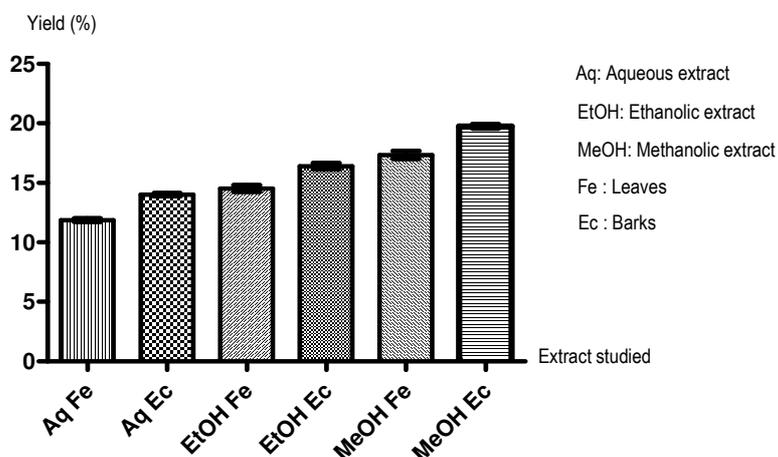


Figure 1: Yield of *T. catappa* extracts

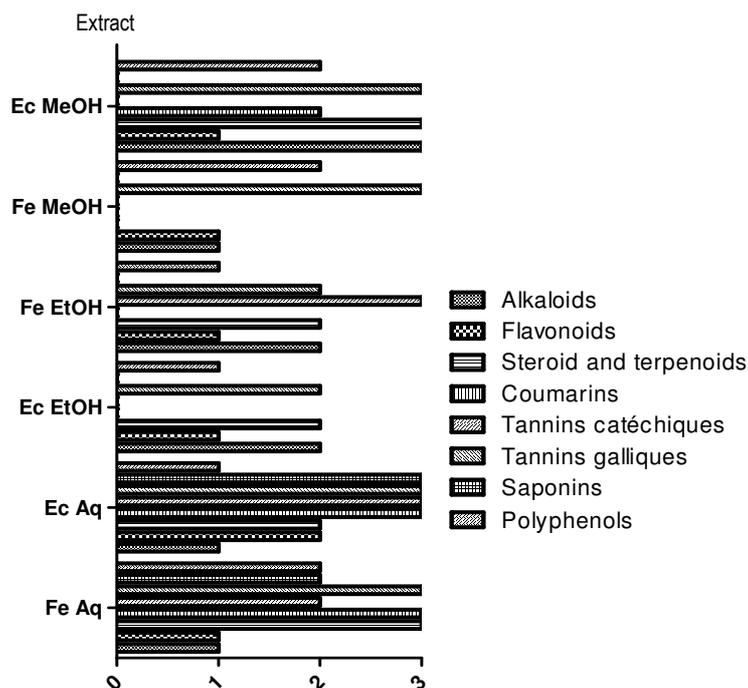


Figure 2: Screening Phytochemical of the extracts of the leaves and barks of *T. catappa*

Antibacterial activity of the crude extracts: All the extracts tested showed antibacterial activity against at least one of the bacterial strains tested with inhibition zones ranging from 0 to 15.4 ± 0.30 mm. One noticed that the susceptibility of the bacteria to the crude extracts of the leaf and bark of *T. catappa* based on inhibition zone diameters varied according to the microorganism, the results are reported in Table 1. In assays disk diffusion, extracts tested showed variable degrees of antimicrobial activities against the tested organisms. The best activity (8.67 ± 0.33 to 15.4 ± 0.30 mm) has been demonstrated by the methanolic extract of bark, and least, by aqueous extract of leaf (0 to 10.00 ± 0.58 mm) Table 1. An inhibition zone diameter (≥ 11 mm) was chosen as a break-point of bacterial susceptibility of the extracts and the antibiotic. Ceftazidime ($30 \mu\text{g}$) and cefoxitin ($30 \mu\text{g}$) were used as positive control and had a diameter of inhibition zone of 13.33 ± 0.88 to 30.67 ± 0.67 mm. It was important to note that the methanolic extract was active against the Gram-positive and all Gram-negative bacteria used in this study at the lowest concentration tested and the pattern of activity observed was such that the zone

diameter of inhibition for each organism was directly proportional to the concentration of the extract used. The Gram-positive bacteria (*S. aureus*) were the most sensitive while the Gram-negative bacteria (especially *Klebsiella pneumoniae*) were more resistant. The bark extracts generally showed a better activity against the test organisms compared to the leaf extracts. The difference is significant ($P < 0.0001$).

Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC)

MIC and MBC values were ranged from 0.52 to 41.67 mg/ml and 1.56 to > 100 mg/ml respectively. The bactericidal and bacteriostatic effect of the extracts was determined using the ratio MBC/MIC (Table 2 and Table 3). The result showed that *Klebsiella pneumoniae* had the highest MIC (41.67 mg/ml) and MBC (> 100 mg/ml) with the aqueous extract of leaf, while the lowest MIC of 1.56 mg/ml was shown by *Staphylococcus aureus* with the methanolic extract of bark. The MIC and MBC values were generally lower for the bark extracts against the test organisms compared to those of the leaf extracts.

Table 1: Zone of inhibition (mm) of the extracts of the leaf of *T. catappa* and céfoxitine and ceftazidime against *E. coli*, *K. pneumoniae* and *S. aureus*

Extract Bacterial strain	Leaves (100 mg/ml)			Barks (100 mg/ml)			Caz/Fox
	Aqueous	Ethanol	Methanol	Aqueous	Ethanol	Methanol	
<i>E. coli</i>	6.67 ± 0.33	8.67 ± 0.33	10.67 ± 0.67	10.67 ± 0.17	11.67 ± 0.17	13.67 ± 0.33	30.00 ± 0.58
<i>E. coli</i> BLSE	5.67 ± 0.33	7.33 ± 0.33	9.33 ± 0.33	9.17 ± 0.17	10.83 ± 0.44	12.17 ± 0.17	14.33 ± 0.33
<i>E. coli</i> ATCC	6.83 ± 0.17	9.67 ± 0.33	11.00 ± 0.58	10.83 ± 0.17	11.67 ± 0.33	13.33 ± 0.33	31.67 ± 0.33
<i>K. pneumoniae</i>	5.17 ± 0.17	7.00 ± 0.58	9.67 ± 0.88	8.83 ± 0.17	10.33 ± 0.33	12.33 ± 0.33	26.33 ± 0.33
<i>K. pneumoniae</i> BLSE	–	6.33 ± 0.33	8.67 ± 0.33	6.50 ± 0.29	8.67 ± 0.17	10.83 ± 0.17	13.33 ± 0.88
SASM	9.83 ± 0.17	11.67 ± 0.33	13.67 ± 0.33	11.33 ± 0.33	13.17 ± 0.17	15.33 ± 0.33	30.67 ± 0.67
SARM	7.33 ± 0.33	10.67 ± 0.33	12.83 ± 0.17	9.83 ± 0.17	11.67 ± 0.33	13.33 ± 0.33	25.67 ± 0.88
<i>S. aureus</i> ATCC	10.00 ± 0.58	11.00 ± 0.58	13.33 ± 0.88	11.50 ± 0.29	13.25 ± 0.25	15.4 ± 0.30	30.17 ± 0.44

Table 2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the leaf of *T. catappa*

Bacterial strain	Aqueous				Ethanol				Methanol			
	CMI	CMB	CMB /CMI	Antibacterial activity	CMI	CMB	CMB/CMI	Antibacterial activity	CMI	CMB	CMB/CMI	Antibacterial activity
<i>E. coli</i>	10.42 ± 2.08	41.67 ± 8.33	4	Bactericidal	8.33 ± 2.08	25.00	3	Bactericidal	3.12	8.33 ± 2.08	2.67	Bactericidal
<i>E. coli</i> BLSE	20.83 ± 4.17	50.00	2.4	Bactericidal	12.50	41.67 ± 8.33	3.33	Bactericidal	3.12	6.25	2	Bactericidal
<i>E. coli</i> ATCC	10.42 ± 2.08	41.67 ± 8.33	4	Bactericidal	5.21 ± 1.04	25.00	4.79	Bacteriostatic	12.50	12.50	1	Bactericidal
<i>K. pneumoniae</i>	12.50	50.00	4	Bactericidal	16.67 ± 4.17	41.67 ± 8.33	2.50	Bactericidal	10.42 ± 2.08	12.50	1.20	Bactericidal
<i>K. pneumoniae</i> BLSE	41.67 ± 8.33	> 100	-	Bacteriostatic	25.00	100	4	Bactericidal	12.50	41.67 ± 8.33	3.33	Bactericidal
SASM	4.16 ± 1.04	8.33 ± 2.08	2	Bactericidal	4.16 ± 1.04	6.25	1.50	Bactericidal	1.56	1.56	1	Bactericidal
SARM	6.25	12.50	2	Bactericidal	6.25	12.50	2	Bactericidal	4.16 ± 1.04	6.25	1.50	Bactericidal
<i>S. aureus</i> ATCC	4.16 ± 1.04	6.25	1.50	Bactericidal	3.12	4.16 ± 1.04	1.33	Bactericidal	1.56	1.56	1	Bactericidal

Table 3: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the bark of *T. catappa*

Bacterial strain	Aqueous				Ethanol				Methanol			
	CMI	CMB	CMB/CMI	Antibacterial activity	CMI	CMB	CMB/CMI	Antibacterial activity	CMI	CMB	CMB/CMI	Antibacterial activity
<i>E. coli</i>	6.25	20.83 ± 4.17	3.33	Bactericidal	4.16 ± 1.04	12.50	3	Bactericidal	1.30 ± 0.26	3.12	2.40	Bactericidal
<i>E. coli</i> BLSE	12.50	20.83 ± 4.17	1.67	Bactericidal	8.33 ± 2.08	12.50	1.50	Bactericidal	4.16 ± 1.04	12.50	3	Bactericidal
<i>E. coli</i> ATCC	6.25	20.83 ± 4.17	3.33	Bactericidal	3.12	12.50	4	Bactericidal	1.56	3.12	2	Bactericidal
<i>K. pneumoniae</i>	12.50	41.67 ± 8.33	3.33	Bactericidal	6.25	12.50	2	Bactericidal	4.16 ± 1.04	5.21 ± 1.04	1.25	Bactericidal
<i>K. pneumoniae</i> BLSE	25.00	> 100	-	Bacteriostatic	12.50	20.83 ± 4.17	1.67	Bactericidal	6.25	10.42 ± 2.08	1.67	Bactericidal
SASM	3.12	10.42 ± 2.08	3.33	Bactericidal	2.08 ± 0.52	3.12	1.50	Bactericidal	0.52 ± 0.13	1.56	3	Bactericidal
SARM	8.33 ± 2.08	16.67 ± 4.17	2	Bactericidal	3.12	12.50	4	Bactericidal	1.30 ± 0.26	4.16 ± 1.04	3.20	Bactericidal
<i>S. aureus</i> ATCC	3.12	12.50	4	Bactericidal	2.08 ± 0.52	3.12	1.50	Bactericidal	0.78	1.56	2	Bactericidal

DISCUSSION

The development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances. Higher plants produce diverse secondary metabolites with different biological activities. These natural compounds may be a source of compounds with antimicrobial effects and therefore possible candidates for the development of new antibacterial agents (Adefuye et al., 2007, Lagnika et al., 2012). In our study, aqueous, ethanol, and methanol extracts of leaves and bark of *T. catappa* were tested for the presence of antimicrobial activity against 11-gram positive and gram-negative bacteria implicated in infectious diseases. The majority of traditional healers use water to extract active compounds from this plant, because water is not harmful to humans and is generally cheap and easily acquired. However, successful isolation of compounds from plant material is largely dependent on the type of solvent used in the extraction process (Masoko et al., 2008). Use of water alone leads to difficulties in isolating non-polar active compounds. The current study, observed that methanol and ethanol were good solvents for extraction with yield of $19.75 \pm 0.17\%$ and $16.39 \pm 0.26\%$ respectively while water was the least $11.87 \pm 0.13\%$. The barks and the methanolic extract have given better yield. The results correspond to those of Ackah et al (2008) and Masoko et al (2008) which have respectively shown a great concentration of the active principals with the methanol. That justifies the fact that the methanolic extract of the barks presented a better activity than most of the tested strains. ($P < 0.0001$) *S. aureus* was the most sensitive strain and *Klebsiella pneumoniae* ESBL was not the least sensitive strain. For an extract considered, comparatively to the control inoculum there

CONCLUSION

This study has allowed us to bring out the antibacterial activity of *Terminalia catappa* and to compare the antibacterial potential of the barks and the leaves of this plant against the multiresistant bacterial strains. Comparatively, barks have shown a good activity than leaves and the methanolic extract of barks has shown better activity with a bactericidal effect upon all the tested strains. This activity was dose dependent. The

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was a progressive decrease in the thickness of the layer of colonies on the streak but also a decrease in the number of colonies from low concentrations to high concentrations; thus reflecting a dose-dependent sensitivity. These results have showed also that all extracts were bactericidal for all the tested strains ($MBC/MIC \leq 4$, Marmonier, 1990). It appears from the analysis of results, that the most bactericidal extract was the methanolic extract of barks with a MBC of 1.56 mg/ml while the least bactericidal extract was aqueous extract of leaves because of its higher value of MBC (> 100 mg/ml). Moreover, some settled report established on the basis of the minimal bactericidal concentration confirm that the barks are better in activity than the leaves, on the other hand the methanolic extract is more active than the ethanolic extract and itself more active than the aqueous extract. Similar results were also shown by Pawar and Pal, 2002; Babayi et al., 2004, Nair Sumitra and Chanda, 2008; and Manzur et al, 2011). Moreover, the Gram-positive bacteria were more susceptible than Gram-negative bacteria. This is in agreement with previous reports that plant extracts are more active against Gram-positive bacteria than Gram-negative bacteria (Prashanth Kumar et al, 2006). The extracts of the barks and leaves of *T. catappa* studied were found to contain more of the following phytochemical compounds flavonoids, Alkaloids, steroid and terpenoids, Gallic, tannis, saponins and coumarins with variable concentration according to the organ and extract. The antibacterial activity of this medicinal plant may therefore be due to the presence of flavonoids, tannis, saponins, steroid and terpenoids (Babayi et al., 2004, Fofana, 2004, Ackah et al, 2008) and hence, the use of this plant in the anti-infectious and anti-diarrhea treatment.

bark of this plant may provide novel or lead compounds, which could become starting materials for the synthesis of new drugs. Therefore, further studies to isolate and characterize the active compounds are essential. This study thus can also justify the traditional use of *Terminalia catappa* in the treatment of many diseases.

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