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Antibacterial and Antioxidant Activities of Hydro-Ehanol Extracts of Barks, Leaves and Stems of *Annona muricata*

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Abstract The purpose of this study was to evaluate the *in-vitro* antibacterial activity of hydro-ethanol extracts of stems, barks and leaves of *Annona muricata* against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabillis* and *Bacillus cereus*, followed by the evaluation of their antioxidant activities. The sensitivity of the bacteria to extracts was evaluated by the well diffusion method and the inhibition parameters of the bacterial growth were determined by the micro-dilution assay. The Minimum Bactericidal concentrations (MBC) obtained were between 6.25 and 25.00 mg/ml. *Pseudomonas aeruginosa* was the most sensitive strain with a MBC value of 6.25 mg/ml obtained from leaves extract. The antioxidant activity was evaluated using1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and ferric reducing antioxidant power (FRAP) assay. Hydro-ethanol extract of barks exhibited the highest DPPH scavenging activity of 0.010 ± 0.004 g/mg and the highest reducing power with ascorbic acid equivalence of 49.45 ± 0.067 mg EAA/gw. This extract has presented only the highest phenolic content 25.00 ± 1.070 mg EAA/gw. The phytochemical screening revealed that the plant contain bioactive compounds such as steroids, triterpenes and alkaloids. These results confirm the use of this plant in traditional medicine for the treatment of bacterial infections and diseases associated to oxidative stress such as diabetes.

Keywords: *Annona muricata, Antibacterial susceptibility, Antioxidant activity, Bioactive compounds*

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1. Introduction

Microbial infections cause around 15 million of deaths every year all over the world, among which 70% are caused by bacterial infections [1]. The situation remains more critical in low and middle income countries where they represent around 45% of death [2]. In Cameroun, bacterial infections remain one of the main causes of morbidity and mortality. According to the OMS estimations (2010) [1], they are the cause of 13 less than five years infants' deaths out of 20 hospitalizations. On the other way, there is increase in bacterial resistance thus constituting a brake for the mastery of these infections. Further, during bacterial infections, there is the production of free radicals which although can be managed by the antioxidant defense system under normal physiological conditions, can increase during infection and give rise to oxidative stress. This state of stress are involved in many diseases like diabetes as a starting factor or associated to complications. Faced with these problems, there is the necessity to search for new therapeutic agents with a broad spectrum of activity, able to fight bacterial infections and attenuate or delay oxidative process.

Plants are considered like the crude materials necessary for the search of new bioactive compounds for the production of future medicines [3,4]. According to the OMS [5] estimations, more than 80% of African population uses traditional medicine for their health problems. Moreover, around 25% of prescriptions are plants based, and 60 to 70% of antibacterial and anticancer are natural substances [6]. Africa and Cameroun in particular possesses a rich flora, accessible and available. *Annona muricata* is a plant of Cameroonian pharmacopeia used in the treatment of many diseases among which parasitic infections. All the different parts of this plant are used in traditional medicine. Some research works realized *in-vitro* has demonstrated the antimicrobial [7], insecticidal [8], antiplasmodial [9] and antifungal properties [10] of this plant against pathogenic agents. Compounds like Annonacine and muricine, isolated from this plant, have shown antimicrobial activity against mosquito larvae [11]. *Annona muricata* constitutes thus a potential source of therapeutic compounds to be explored. The aim of this study was to evaluate the antibacterial and antioxidant activities of hydro-ethanol extracts of barks, leaves and stems of *Annona muricata*.

2. Material and Methods

2.1. Identification and Collection

The barks, stems and leaves of *Annona muricata* were harvested at Yaoundé the 06 January 2014 and the botanical identification was done at the Cameroon National Herbarium by comparison with the specimen N°32879 /HNC.

2.2. Bacterial Strains

Bacterial strains were made of 5 clinical isolates among which 3 Gram negatives (*Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus mirabilis*) and 2 Gram positive (*Staphylococcus aureus, Bacillus cereus*). Theses isolates were gratefully given by Centre Pasteur (Yaoundé) and stored at +4°C on MHA medium.

2.3. Extract Preparation

The samples of different parts of *Annona muricata* were dried in the shade and away from moisture during 3 weeks. These samples were crushed separately using an electrical grinder. The powdered samples were macerated in ethanol/water mixture (8:2) for 72h. The macerate obtained was filtered through Whatman No.1 filter paper and the filtrate was concentrated using a rotary evaporator. The extract obtained were weighed and stored at +4°C in the refrigerator. Extraction yield were determined according to the formula:

$$Extraction\ yield(\%) = \frac{Masse\ of\ extract}{Mass\ of\ powder\ macerated} \times 100$$

2.4. Phytochemical Screening

Phytochemical screening was carried out according the standard procedures described by Harbone [12] and Sofowora [13].

2.5. Polyphenols Content

The polyphenols content of *Annona muricata* extracts was determined by the spectrophotometric method using the Folin-Ciocalteu reagent assay [14]. Volumes of 1817 µl of distilled H_2O, 23 µl of the extract, 115 µl of Folin-Ciocalteu (1:10) and 345 µl of sodium carbonate Na_2CO_3 were successively added in a test tube. The mixture was vortexed and then incubated for two hours. After incubation, the absorbance was measured at 765 nm. The standard used was ascorbic acid solution and results were expressed as milligram equivalent of ascorbic acid/gram dry weight.

2.6. Antibacterial Activity Evaluation

2.6.1. Preparation of the Bacterial Inoculum

From 24h culture of each clinical bacterial culture on Mueller Hinton Agar medium (MHA), the bacterial inoculum was prepared at 0.5 McFarland in physiological saline (0.9% NaCl solution), and adjusted at 10^6 CFU/ml by dilution [14].

2.6.2. Susceptibility Tests

The preliminary susceptibility tests of the bacterial strains to the various extracts were carried out as recommended by Clinical and Laboratory Standards Institute [16]. Hundred microliters (100 µl) of each bacterial inoculum was inoculated on Mueller Hinton agar. The Petri dishes were then allowed to dry at ambient temperature under a fumes cupboard for 15 min. Six millimeters (6 mm) wells were bored in the agar and the bottom of each well plugged with a drop of Mueller Hinton agar to limit the diffusion of the extracts from below. Fixed volumes of 50 µL of the tested substance were then introduced in each well. After a pre-diffusion time of 15 min of the test extracts at ambient temperature, the Petri dishes were incubated at 37°C for 24 h. The inhibition diameters round each well was measured using a sliding caliper. Each test was done in triplicate and the result express in the form of average ± standard deviation.

2.6.3. Evaluation of Inhibition Parameters

The inhibition parameters of bacterial growth were evaluated according to the modified M27-A9 broth microdilution method described by National Committee for Clinical Laboratory Standards [17]. This involved preparing double dilutions of test extracts in 100 µl of glucose Nutrient Broth into the wells of a microtiter plates. The range of final concentrations tested were 0.39 to 25.0 mg/ml for each plant extract and 0.039 to 2.50 mg/ml for gentamycin. Each serial dilution was performed in triplicate. Hundred microliters (100 µl) of bacteria inoculum were distributed to all the wells of the microtiter. A line of the plate without plant extract was served as a control for the growth of the organism (negative control) and another without plant extract and inoculum was served as sterility testing medium (positive control). The plates were incubated at 37°C for 24 hours. After incubation, microbial growth was revealed by using 2,3,5-Triphenyltetrazoliumchloride (TTC). Forty microliters (40 µl) of TTC (0.2 mg/ml) was introduced into all the wells with the exception of those that were used for the MBC. After 30 min of incubation, the change in color to red indicated bacterial growth. The minimum inhibitory concentration (MIC) was retained as the lowest concentration for which no color change of the medium was observed. The minimum bactericidal concentration (MBC) was determined by subcultures. Fifty microliters (50 µl) of the contents of wells with concentration greater than or equal to MIC were introduced in 150 µl of Nutrient broth medium. After 48h of incubation at 37°C, subcultures for which there was no resumption of growth corresponded to MBC.

2.7. Antioxidant Activity Evaluation

2.7.1. DPPH Radical Scavenging

The ability of *Annona muricata* extracts to scavenge the free radical DPPH was evaluated according the method of Brand-Williams et al. [18]. Briefly, 8 mg/ml of extract in water was prepared and further dilutions namely: 4, 2, 1, 0.5, 0.25 and 0.125 mg/ml were realized. Fifty microliters (50 µl) of each one was separately mixed with 1950 µl of DPPH solution in methanol (0.004 w/v %), to obtain concentrations ranging between 0.05 and 0.00156 mg/ml. This was incubated at the shade of light for 2 hours. The ascorbic acid, in the same concentrations was served as positive control. After incubation, the optical density was

written at 515 nm. The mixture without extract sample was used as blank and just spiked with 50 µl of methanol. The absorbance was written in the times: 0, 15, 30, 45, 60, 75, 90, 105 and 120 min. All the tests were done in triplicate. The SC_{50} (needed concentration to scavenge 50% of free radicals) was determined graphically. Scavenging concentration (SC), EC_{50} (concentration needed to scavenge 50% of DPPH) and AP (antiradical power) were determined according to the following formulas:

$$SC = \frac{\left(Absorbance\ of\ positif\ control - Absorbance\ of\ test\right)}{Absorbance\ of\ positve\ control} \times 100$$

$EC_{50} = SC_{50}$ / Concentration of DPPH AP= 1/ EC_{50}

2.7.2. FRAP: Ferric Reducing Antioxidant Power

The reducing ability of Fe^{3+} to Fe^{2+} of *Annona muricata* extracts was evaluated according the method described by Benzie and Strain [19]. A volume of 50 µl of test extract was introduced in 1950 µl of FRAP solution (40 mM of 2,4,6-tripyridyl-*s*-triazine in Hcl, 20Mm $FeCl_3$, 300 mM acetate buffer at pH 3.6, in ratio 1:1:10) to obtain concentrations of extract ranging between 0.025 and 0.00078 mg/ml. Incubation was carried out at the shade of light for 30 min and optical density was measured at 593 nm. The tests were done in triplicate. The results were expressed in milligram of test extract equivalent in ascorbic acid per gram of dry weight (mg EAA/gw).

3. Results

3.1. Phytochemical Contents

Table 1 below summarizes the extraction yields obtained from the different parts of *Annona muricata* as well as the results obtained for the phytochemical screening. The extraction yield obtained from the hydro-ethanol extracts of *Annona muricata* are ranged between 3.82 % (stems) and 5.46 % (leaves). The results obtained from the phytochemical screening show that the plant is rich in many bioactive compounds among which are polyphenols, flavonoids, tannins, steroids, triterpenes, alkaloids and cardiac glycosides.

Table 1. Extraction yield and phytochemical content

Part of plant	Barks	Leaves	Stems
Extraction yield	5.14 %	5.46 %	3.82 %
Polyphenols	+	+	+
Flavonoïds	+	+	+

3.2. Polyphenols Content

The polyphenolic content, expressed in milligram equivalent ascorbic acid per gram of dry weight (mg EAA/gw), was determined using the standard curve obtained from known concentrations of ascorbic acid, used as the reference. The total polyphenols content (Table 2) of hydro-ethanol extracts of *Annona muricata* was carried out using the Folin-Ciocalteu reagent. The results obtained show that barks had the highest polyphenols content of 25.0 ± 1.07 mg EAA/gw, follow by stems (24.0 ± 1.03 mg EAA/gw) and by leaves (23.0 ± 0.90 mg EAA/gw).

Table 2. Total phenolic content (Folin-Ciocalteu assay)

Plant parts	Barks	Leaves	Stems
Polyphenols content (mg EAA/gw)	25.0 ± 1.07	23.0 ± 0.90	24.0 ± 1.03

3.3. Evaluation of Antimicrobial Activity

3.3.1. Antimicrobial Activity

The inhibition diameters obtained from crude extract of the bark ranged from 13.3 ± 2.30 mm to 21.0 ±1.70 mm; those obtained from leaves extracts, from 8.00 ± 0.20 mm to 14.7 ± 1.30 mm and from stem extract, 8.00 ± 0.10 mm to 18.0 ± 1.70 mm. The solvent for dissolution of the extracts, Tween 10% had no effect on the bacterial growth (absence of inhibition zone around the well).

Table 3. Inhibition diameters on tested strains

Bacterial strains	Inhibition diameters			
	Barks	Stems	Leaves	Gentamycin
Staphylococcus aureus	12.3 ± 1.10	10.7 ± 0.50	8.00 ± 0.20	24.5 ± 2.30
Pseudomonas aeruginosa	21.0 ± 1.70	18.0 ± 1.70	18.3 ± 1.50	21.7 ± 2.80
Klebsiella pneumoniae	16,7 ± 1.50	14.7 ± 4.50	11.7 ± 0.50	30.0 ± 2.60
Proteus mirabillis	13.3 ± 2.30	11.0 ± 0.10	9.70 ± 1.52	18.7 ± 1.52
Bacillus cereus	14.7 ± 1.50	8.00 ± 1.00	14.7 ± 1.30	19.0 ± 0.76

3.3.2. Inhibition Parameters

The results obtained from inhibition parameters (Table 4) showed that MICs determined are ranged between 0.56 and 12.5 mg/ml and MBC between 6.25 and 25.0 mg/ml. According to Fauchère and Avril [20] when the MBC of an antibiotic on a given strain is close to the MIC ($1 \leq MBC/MIC \leq 2$), the antibiotic is described as being bactericidal. On the other hand, when these values are relatively distant, ($4 \leq MBC/MIC \leq 16$), the antibiotic is known to be bacteriostatic. Lastly if the MBC/MIC >16, it is described tolerant.

3.3.3. DPPH Assay

The ability to scavenge the radical DPPH was evaluated with a spectrophotometer at 515 nm. Antiradical parameters (SC_{50}, EC_{50}, PA) obtained are in Table 5 below. The DPPH scavenging ratio was positively and significantly correlated with concentration. The correlation coefficients

were 0.8535, 0.8907 and 0.9876 for the barks, leaves and stems hydro-ethanolic extracts, respectively. The results obtained from DPPH scavenging activity of different extracts are mentioned in Figure 1 below.

Table 4. MIC, MBC and MBC/MIC ratio of extracts/Gentamycin

Extracts/ Standard	Inhibition parameters	Bacterial strains				
		SA	BC	PA	KP	PM
Barks	MIC	12.5	12.5	6.25	6.25	12.5
	MBC	12.5	25	12.5	ND	ND
	MBC/MIC	1	2	2	ND	ND
Leaves	MIC	12.5	12.5	6.25	12.5	ND
	MBC	25.0	25.0	6.25	ND	ND
	MBC/MIC	2	2	1	ND	ND
Stems	MIC	1.56	3.12	3.12	12.5	12.5
	MBC	25.0	12.5	12.5	ND	12.5
	MBC/MIC	16	4	4	ND	1
Gentamycin	MIC	0.12	0.01	0.06	0,06	0.01
	MBC	0.12	0.03	0.06	ND	0.12
	MBC/MIC	1	2	1	ND	8

Legend: SA = Staphylococcus aureus, BC = Bacillus cereus, PA = Pseudomonas aeruginosa, KP = Klebsiella pneumoniae, PM = Proteus mirabillis, ND = Not Determined

Table 5. DPPH radical scavenging parameters

	SC_{50} (g/l)	EC50 (mg/gDPPH)	AP (g/mg)
Barks	0.0036 ± 0.001	90 ± 0.027	0.01 ± 0.004
Leaves	0.011 ± 0.0003	290 ± 0.0078	0.0034 ± 0.0009
Stems	0.0046 ± 0.00007	116 ± 0.02	0.0089 ± 0.0001
Ascorbic acid	0.0063 ± 0.001	157.50 ± 0.02	0.0063 ± 0.0003

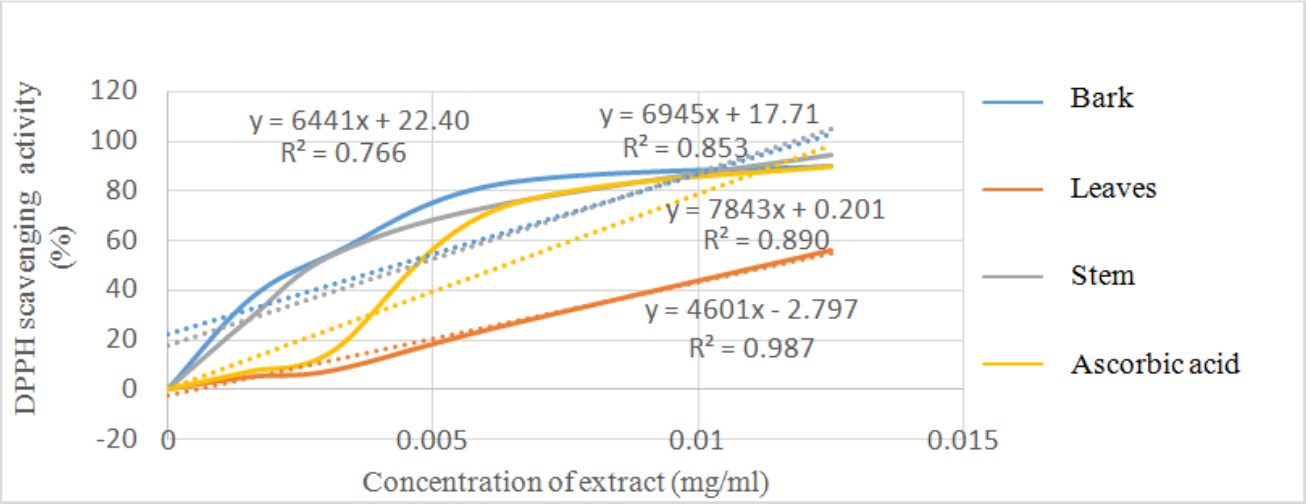

Figure 1. DPPH scavenging activity as a function of extract concentrations

Table 6. Equivalence in mg of ascorbic acid per gram of dry weight of extract at different concentrations tested

Concentrations of ascorbic acid (mg/ml)	Equivalence of extracts in mg of ascorbic acid		
	Barks	Leaves	Stems
0.00078	2.09±0.008	0.38±0.015	0.25±0.08
0.00156	6.68±0.02	1.13±0.008	0.34±0.053
0.003125	14.49±0.004	3.99±0.024	0.878±0.043
0.00625	25.49±0.35	8.4±0.02	2.36±0.13
0.0125	41.69±0.07	16.73±0.018	6.26±0.27
0.025	49.45±0.067	33.58±0.067	14.34±0.57

3.3.4. FRAP Assay

The regression curve of ascorbic acid (y = 2.5746 x + 0.0122; r^2 = 0.9607) has permitted the expression of the reducing power of extract as equivalence in mg ascorbic acid per gram of dry weight (Table 6).

4. Discussion

Results obtained from phytochemical screening of *Annona muricata* extracts (Table 1) revealed that this plant contain metabolic groups like polyphenols, phenols, tannins, triterpenes, steroids, alkaloids, flavonoids and cardiac glycosides. These bioactive compound groups have demonstrated in many studies theirs *in-vitro* and *in-vivo* antibacterial and antioxidant activities. These results are in accordance with the findings of Solomon-wisdom et al. [21]. Results obtained from total phenolic content evaluation revealed that phenolic concancentrations were 25.0 ± 1.07, 24.0 ± 1.03 and 23.0 ± 0.90 mg EAA/gw for the barks, stems and leaves hydro-ethanol extracts, respectively.

Preliminary susceptibility tests (Table 3) showed effective inhibition of microorganisms growth. At 100 mg/ml, the inhibition diameters obtained from barks hydro-ethanol extract were ranged between 12.3 and 21.0 mm, those obtained from leaves extract varied from 8.00 to 18.3 mm and those obtained from the stem extract were ranged between 8.00 and 18.0 mm. This difference could be due to the fact that, the sensitivity of a microorganism to an extract depends not only on the extract but on the microorganism itself [22]. Nevertheless, these antibacterial activities were less than that obtained with the reference antibiotic, gentamycin. The most sensitive strain was *Pseudomonas aeruginosa* with barks hydro-ethanolic extract (21.0 ± 1.70 mm). The results of these susceptibility tests are in accordance with those obtained by Vijayameena et al. [23] who had proven the susceptibility of bacteria such as *Staphylococcus aureus, Bacillus, Pseudomonas aeruginosa, Klebsiella pneumonia* to barks and leaves extracts of *Annona muricata*. This justifies the traditional uses of this plant for the treatment of bacterial infections.

With regards to the inhibition parameters (Table 3), the MIC ranges from 1.56 to 12.50 mg/ml, and the MBC between 6.25 to 25.00 mg/ml. The ratio MBC/MIC was determined and according to the classification made by Fauchère and Avril [20], majority of *Annona muricata* extracts were bactericidal on the strains tested ($1 \leq CMB/CMI \leq 2$). This justifies the traditional uses of this plant to treat bacterial infections. The antibacterial activity of these extracts can be ascribed to the presence of secondary metabolites. Cowan [24] had demonstrated that phenolic compounds had great antibacterial activity due to their mechanism of toxicity that manifests through the chelation of metallic ions and sequestration of substances necessary for the growth of bacteria. Phenolic compounds such as flavones and flavanols have demonstrated their antibacterial potential and their capacity to scavenge free radicals such as hydroxyl radical and nitrites. Terpenoids has been cited to induce a destruction of the microorganism's membrane through a lipophilic action; they have the properties to precipitate microbial proteins or viral and heavy metals [25]. Flavonoids antimicrobial activity could be due to their ability to complex with polypeptides of the microbial cell wall [26]. Alkaloids have the capacity to link with bacterial DNA [27].

Antioxidant evaluation by the DPPH assay revealed that *Annona muricata* extracts enclosed antioxidant activity. The EC_{50} of the barks, stems and leaves extracts are respectively 0.09 ± 0.027, 0.116 ± 0.02 and 0.29 ± 0.0078 mg/g of DPPH. Previous studies had shown the influence of organ of plant extracted on yield of total phenolic content and antioxidant activity of the extracts. The higher radical scavenging activity of barks extract could be due to the high content in phenolic compound compared to stems and leaves extracts. Several studies have shown the relationship between the antioxidant activity and total phenolic compounds [28,29]. Phenolic compounds like flavonoids, due to their chemical structure, are ideal donors of hydrogen to the DPPH radical [30]. Secondary metabolites like steroids and alkaloids found in *Annona muricata* have also demonstrated their antioxidant properties in several studies.

The results from the ferric reducing assay potential (FRAP) reveal that the bark had the highest reducing capacity followed by the leaves and stem. These results were consistent with the findings of many research groups who reported such positive correlation between total phenolic content and antioxidant activity [28,31]. This result reveals the electrons donating capacity possessed by the polyphenols due to the number, configuration and glycosylation of hydroxyl groups [32]. It could also be due to the presence of others metabolites groups like alkaloids which are compounds known for their antioxidant power [33,34]. Many studies have demonstrated that these compounds are antioxidants; possessing hydrogen atoms and a single electron transfer mechanism [35].

These results indicate that compounds present in the hydro-ethanolic extracts of *Annona muricata* are capable of reducing DPPH radicals and also able to reduce ferric ions.

Based on the *in vitro* antibacterial and antioxidant activities shown by the extracts of *Annona muricata*, this plant has been proven to contain several bioactive components which could justify the renewed interest for the exploitation of this natural resource in the development of antibacterial drugs in order to overcome the problem of narrow spectrum of activity of orthodox antibiotics.

5. Conclusions

The present study, overall results showed that *Annona muricata* extracts possess both antimicrobial and antioxidant properties. Phytochemical screening reveals that this plant contains bioactive metabolites like polyphenols, tanins, triterpenes, steroids, alkaloids, flavonoids and cardiac glycosids. The antibacterial and antioxidant activities of these metabolites groups have been demonstrated in several authors. This study is a contribution to the search of new medicine in the treatment of bacterial infections and diseases caused by oxidative stress.

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