



Antibacterial and toxicological evaluation of the ethanol leaf extract of *Anthonotha macrophylla*

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ABSTRACT

Introduction: The leaves of *Anthonotha macrophylla* which are widely used in folkloric medicine in Africa were evaluated for antibacterial activity and its toxicological profile.

Methods: Antibacterial activities of the leaves against clinical strains of *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Streptococcus pyogenes* and *Bacillus subtilis* were compared with cefoxitin. Toxicological profiles were determined following the administration of 100, 200 and 400 mg/kg of the leaf extract and distilled water, as control, to male Albino rats for 28 days. Biochemical and hematological indices and body weights were determined and the vital organs were examined.

Results: The ethanol leaf extract of *A. macrophylla* demonstrated antibacterial activity against the pathogenic bacteria tested. Toxicological investigation of the leaf extract showed no significant alterations in hematological and biochemical indices, and the vital organs as well. The LD₅₀ in rats was more than 5000 mg/kg.

Conclusion: Findings suggest that the ethanol leaf extract of *A. macrophylla* possess good antimicrobial activity which supports the traditional use of this plant across Africa. The plant is also considered safe following oral exposure after 28 days.

Implication for health policy/practice/research/medical education:

Anthonotha macrophylla ethanol leaf extract significantly demonstrated broad-spectrum antibacterial activity and nontoxic effect. Hence, this plant can be considered as a candidate for bioassay-guided and isolation of compounds which could possibly be developed into new lead structures for drug development programs against diseases.

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Introduction

The use of plants for medicinal purposes is an age long practice which has given rise to a number of new therapeutic agents (1-3). Traditional medicine remains the mainstay of health care system especially in developing countries where orthodox medicines are regularly used. Medicinal plants are being employed globally for the treatment of different diseases which could not be properly managed with conventional agents (4). However, these plants are continually being evaluated for their bioconstituents and pharmacological activities. More so, researchers are making progressive efforts to determine the advantages of herbal medicines in modern science aimed at adopting

efficient medical practice and overcoming the harmful ones (5).

Anthonotha macrophylla P. Beauv (family Leguminosae-Caesalpinioideae) is one of the plants used by the locals in Akwa Ibom and Cross River States, Nigeria to treat a number of health challenges. It is one of the species in the genus *Anthonotha*, a shrub of 4-20 m in height grown in the wild or uncultivated farmlands. Its vernacular names are 'nya' by the Ibibios and Efiks, 'ububa-iepa' by the Igbos and 'abara' by the Yorubas (6). The plant originated from Liberia but has spread to other parts of Tropical African countries including Cameroon, Gabon and Zaire in the West-Central; Nigeria and Ghana in the

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West; and Angola in the South. The leaves of the plant are particularly used in folkloric medicine with claims of its effectiveness in the treatment of pain, boils, malaria, worm infestation, gonorrhoea, dysentery, yellow fever and diarrhoea. However, there is limited scientific evidence on most of such usage. The antimalarial activity of the leaf extract has been scientifically established (7). However, till date studies on antibacterial and toxicological profile of the ethanol leaf extract of *A. macrophylla* have not been reported. Hence, the present study evaluated the possible antibacterial potential of the plants' leaf extract and its safety in rats.

Materials and Methods

Collection and preparation of plant materials

The leaves of *A. macrophylla* were collected from a farmland in Ikot Ambang village in Ibiono Local Government Area of Akwa Ibom State, Nigeria during the months of February and March 2017. The leaves were authenticated in the Department of Botany, University of Calabar and a specimen with voucher number 736 was deposited in the herbarium of that department for future reference. The leaves were cleaned, air-dried on the surface of the laboratory bench and ground into coarse powder using a hand operated grain mill (Corona®, Columbia).

Preparation of the extract

Five hundred grams of the leaf powder was extracted in ethanol using Soxhlet apparatus. The ethanol extract solution was dried on a water bath at a temperature of 45°C to give a yield of 21.02% w/w on dried weight basis. The extract was reconstituted in distilled water for use during the experiment.

Phytochemical analysis

The presence of bioactive constituents from the ethanol leaf extract of *A. macrophylla* was carried out using standard chemical methods (8,9).

Microbial strains

Six clinical pathogenic bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Bacillus subtilis*, *Streptococcus pyogenes*, *Staphylococcus aureus* and *Salmonella typhi*) were used to evaluate the antibacterial activity of the ethanol leaf extract. All isolates were from the Department of Microbiology, University of Calabar Teaching Hospital, Calabar.

Antimicrobial assay

The antimicrobial activity of the ethanol extract was assayed using disc agar diffusion method of Akuodor et al (10). Before the assay, purity plates of required microbial isolates were isolated and biochemical tests carried out to confirm the identity of the organisms. Furthermore, discrete colonies of the fresh cultures from bacterial isolates were however mixed in 5 mL nutrient broth and

incubated at 37°C for 24 hours. Broth cultures of 0.5 mL isolate containing 10⁵ cfu/mL of organism thoroughly reconstituted and emptied into sterile petri-dish together with 15 mL of Muller-Hinton agar was prepared to the manufacturer's instructions. The content was thoroughly mixed and allowed to solidify. Holes were bored in the plates with a standard sterile cork borer of 8 mm in diameter and the ethanol leaf extract reconstituted in distilled water at concentrations of 50, 100, 150, 200 and 250 µg/mL, solvent blank and cefoxitin (standard antimicrobial drug) were applied in different wells in the culture plates. The studies were carried out in duplicate, the plates were kept for 2 hours for proper mixing of the extract which was then incubated at 37°C for 48 hours. The diameters of zones of inhibition were later measured against the organisms tested. Cefoxitin in concentration of 20 µg/mL was used as a standard drug and distilled water was used as control. The diameter of area of inhibition (antibacterial activity) was then compared with the standard drug and control.

Determination of minimum inhibitory concentration

The method of Andrews (11) was used to determine the minimum inhibitory concentration (MIC) of *A. macrophylla* ethanol leaf extract. Six tubes (sterile) were arranged in a rack and 0.5 mL nutrient broth was each transferred into test tube. Later, the extract was serially diluted to the concentration of 250, 200, 150, 100 and 50 µg/mL. Then, 0.5 mL organism was aspirated and transferred the test tubes containing both the broth and the extract which was incubated at 37°C for 24 hours. The MIC was observed as the lowest concentration of the leaf extract with no visible growth of the test organism.

Experimental animals

Adult male albino rats weighing 184-192 g were sourced from the Department of Pharmacology, University of Calabar and used for the experiments. All animals were housed in plastic cages with wire gauzed tops and saw dust as beddings. They were acclimatized for seven days in laboratory conditions (relative humidity of 50±5 %, temperature 28±2°C and 12 hours of light/ dark cycle) and maintained on the same conditions for the duration of the studies. The rats were fed with pellets (Agro Feeds, Calabar) and clean water *ad libitum*. Handling of the animals was according to the International Guidelines for Care and handling of experimental animals (12).

Toxicological evaluation

Acute toxicity test

Male Albino rats weighing 184-192 g grouped in 4 per cage were orally treated with the test leaf extract (*A. macrophylla*) at doses of 100 mg/kg, 600 mg/kg, 1000 mg/kg, 2000 mg/kg, 3000 mg/kg and 5000 mg/kg (4 rats for each dose). Each group of rats was placed in a test cage for

a 45-minute adaptation period before oral administration of the extract using an orogastric cannula. The animals were first observed for 4 hours and later 72 hours. Toxicity signs and mortality were recorded for each group after the observation period (13). The experimental rats were monitored further for seven days.

Sub-acute toxicity study

The protocol described in Organization Economic Community Development (OECD) guidelines number 407 (14) was adopted but with slight modifications. Twenty-four male albino rats weighed and grouped into 4 with 6 (n=6) rats in each group were used for the study. The treatment groups were administered daily via a gastric cannula – 100 mg/kg, 200 mg/kg and 400 mg/kg body weight of the ethanol leaf extract of *A. macrophylla* respectively, while control rats were given distilled water for 28 days. At the end of experimentation, the rats were anesthetized under chloroform vapour and blood samples collected via cardiac puncture into plain and EDTA containing sample bottles for biochemical and hematological analyses respectively. The organs (liver, kidney, heart and lungs) were removed, weighed and examined microscopically.

Hematologic analysis

Hematological analysis was done with an automated hematology analyzer (Sysmex-XT-1800, Kobe, Japan) in the University of Calabar Teaching Hospital, Calabar, Cross River State, Nigeria.

Biochemical studies

Serum was generated by allowing the blood in the plain bottles to stand for 3 hours at room temperature to ensure complete clotting; centrifuging the clotted samples at 3500 rpm for 10 minutes and the resultant clear serum was aspirated and stored for the biochemical studies.

Biochemical indices were determined with standard ready-to-use reagent kits (Randox Ltd, UK) following the manufacturer's instructions. The indices assessed were liver indices (aspartate aminotransferase - AST, alanine aminotransferase - ALT, alkaline phosphatase - ALP, total proteins/albumin levels and bilirubin levels - total/direct/indirect), kidney indices (electrolytes, creatinine and uric acid).

Organ weights

The rats' vital organs (heart, lungs, liver and kidneys) were excised, cleaned of blood, weighed and examined macroscopically, and their relative weights calculated as: Relative organ weight - ROW (%) = weight of organ / body weight of rat × 100.

Statistical Analysis

The SPSS version 20.0 software was used for data processing and values obtained from descriptive statistics

expressed as means ± SEM. Statistical significance was determined with one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison tests. Differences were considered significant at $P < 0.05$.

Results

Phytochemical constituents

Phytochemicals detected in the ethanol leaf extract of *A. macrophylla* were polyphenols, tannins, flavonoids, saponins, steroids, glycosides, alkaloids, while anthraquinones were absent.

Antibacterial activity

The results of antibacterial activity of the ethanol leaf extract and the MIC for the organisms are presented in Table 1. The leaf extract showed greater activity against *E. coli*, *K. pneumonia*, *S. pyogenes* and *S aureus*. Cefoxitin at concentration of 20 µg/mL, showed activity against all the tested pathogenic bacteria.

Acute toxicity test

The ethanol leaf extract of *A. macrophylla* did not produce any lethality or visible signs of toxicity in rats up to the oral dose level of 5000 mg/kg body weight 24 hours after treatment. Further monitoring for seven days did not still yield mortality or visible toxic signs. Hence, the LD₅₀ value was greater than 5000 mg/kg body weight.

Effects on hematological indices

Table 2 represents the effects of ethanol leaf extract of *A. macrophylla* on hematological indices. There were insignificant alterations in hematological parameters in rats exposed to all doses of the ethanol leaf extract compared to control.

Effects on biochemical parameters

The effects of *A. macrophylla* ethanol leaf extract assessment on biochemical parameters in rats are shown in Table 3. The extract produced non-significant effects on the plasma levels of liver enzymes (ALT, AST and ALP), total proteins and albumin compared to the control group.

Table 1. Antibacterial activity of the ethanol leaf extract and the MIC

Organisms	Inhibition zones of bacteria (mm)		MIC (µg/mL)
	Extract	Cefoxitin	
<i>Staphylococcus aureus</i>	11	12	23
<i>Escherichia coli</i>	20	9	25
<i>Salmonella typhi</i>	8	8	100
<i>Klebsiella pneumonia</i>	16	8	12.5
<i>Streptococcus pyogenes</i>	14	10	27
<i>Bacillus subtilis</i>	10	10	25

MIC, Minimum inhibitory concentration.

Table 2. Effects of ethanol leaf extract of *Anthonotha macrophylla* on rats' hematological indices

Parameters	Control	Doses		
		100	200	400
RBC (x10 ¹² /L)	8.82±2.17	8.62±2.55	9.11±1.71	8.80±2.52
HGB (g/dL)	16.90±2.20	16.62±2.81	17.19±1.91	17.51±2.16
PCV (%)	51.51±0.31	50.18±0.11	52.20±0.22	52.80±0.03
MCV (fl)	32.16±0.33	31.91±0.25	32.06±0.19	31.84±0.54
MCHC(g/dL)	31.29±0.51	30.94±0.88	30.60±0.59	31.23±0.22
WBC (x10 ⁹ /L)	11.43±0.75	11.61±0.51	11.42±0.94	11.72±0.66
PLT (10 ³ /L)	945.11±42.81	944.00±44.1	943.93±50.81	950.03±50.11
Neutrophils (%)	25.03±1.61	25.11±1.33	24.76±2.01	25.56±1.11
Lymphocytes (%)	70.54±11.10	70.33±11.06	70.59±10.9	70.31±12.21
Monocytes (%)	1.06±0.32	0.91±0.42	1.05±0.29	0.94±0.42
Eosinophil (%)	1.23±0.16	1.31±0.11	1.22±0.14	1.30±0.12

Values are means ± SEM (n = 6).

RBC, red blood cells; HGB, hemoglobin; PCV, packed cell volume; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; WBC, white blood cells; PLT, platelets.

Table 3. Effect of ethanol leaf extract of *Anthonotha macrophylla* on rats' liver indices

Parameters	Control	Doses		
		100	200	400
AST (IU/L)	170.1±14.2	173.4±13.2	175.0±6.2	175.6±4.6
ALT (IU/L)	46.5±9.5	47.9±10.7	48.4±6.4	47.6±7.4
ALP (IU/L)	196.71±10.1	198.4±10.1	196.4±11.3	194.3±12.5
TP (g/dL)	6.65±3.2	6.01±4.4	6.61±3.5	6.70±2.4
Alb. (g/dL)	2.75±11.1	2.70±9.2	2.67±8.5	2.10±6.4

Values are means ± SEM (n = 6).

AST, Aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; TP, total protein; Alb, albumin.

Effects on lipid profile

As shown in Table 4, the lipid profile of treated rats was not significantly altered in the ethanol leaf extract treated rats compared to the control group.

Effects on renal indices

Analysis of renal parameters in the extract treated rats showed a non-significant reduction in concentration at all employed doses compared to control group (Table 5).

Effects on relative organ weight of rats

There was no significant difference in the rats' relative

vital organs weights in the extract-treated rats compared with the control group (Table 6).

Discussion

Herbal medications are globally gaining popularity in primary health care with great potential to provide new therapeutic agents. However, there are concerns about the scarcity of scientific information on safety profiles of most of these products (15). The pharmacological actions, beneficial and adverse effects of herbal recipes are attributed to their chemical constituents (16). Different methods are available for obtaining the constituents

Table 4. Effect of leaf extract of *Anthonotha macrophylla* on lipid profile of rats

Parameters	Control	Doses		
		100	200	400
Triglycerides (mmol/L)	0.67±0.15	0.69±0.12	0.73±0.08	0.76±0.14
Total cholesterol (mmol/L)	1.74±5.11	1.72±7.10	1.77±9.01	1.75±13.3
HDL (mmol/L)	0.33±0.71	0.31±0.10	0.34±0.05	0.32±0.11
LDL (mmol/L)	1.11±0.11	1.13±0.12	1.12±0.04	1.14±0.08
VLDL (mmol/L)	0.30±0.05	0.28±0.06	0.31±0.12	0.29±0.12

Values are means ± SEM (n=6).

HDL, high density lipoprotein; LDL, low density lipoproteins; VLDL, very low density lipoprotein.

Table 5. Effect of ethanol leaf extract of *Anthonotha macrophylla* on rats' kidney indices

Parameters	Control	Doses		
		100	200	400
Na (mmol/L)	141.6±10.3	140.9±11.2	142.4±10.6	142.3±15.2
Ca (mmol/L)	2.7±0.3	2.6±0.4	2.7±0.3	2.5±0.7
K (mmol/L)	5.5±1.2	5.4±1.7	5.8±0.6	5.9±1.0
Cl (mmol/L)	96.4±6.3	93.6±7.1	97.0±5.8	96.3±6.8
Phosphate (mmol/L)	2.0±0.3	2.0±0.5	2.1±0.3	2.3±0.1
Creatinine (µmol/L)	42.6±2.1	43.3±1.8	41.6±6.3	43.6±4.0
Urea (mmol/L)	8.3±1.0	8.2±1.5	8.0±2.5	8.4±1.0
Uric acid (mmol/L)	159.3±11.7	160.8±10.6	161.4±9.5	159.9±16.8

Values are means ± SEM (n = 6).

Table 6. Effect of leaf extract of *Anthonotha macrophylla* on albino rats' relative weight of vital organs weight

Parameters	Control	Doses		
		100	200	400
Liver	3.51±0.13	3.50±0.10	3.49±0.25	3.53±0.11
Kidney	0.76±0.02	0.75±0.05	0.77±0.04	0.78±0.03
Heart	0.38±0.01	0.39±0.01	0.40±0.01	0.37±0.03
Lungs	0.57±0.04	0.58±0.05	0.56±0.07	0.57±1.00

Values are means ± SEM (n = 6).

of any medicinal plants. Phytochemical analysis of the ethanol leaf extract of *A. macrophylla* revealed the presence in varied proportions of alkaloids, saponins, tannins, polyphenols, flavonoids, steroids, glycosides and anthraquinones. Initial screening of medicinal plants for secondary metabolites assists the detection of bioactive compounds, which may initiate drug discovery in one hand and possible adverse effects on the other hand (17). These biochemicals may be behind the observed effects in this study.

Antimicrobial properties of medicinal plants are being increasingly reported globally. In this study, the leaf extract exhibited potent activity against the tested pathogenic bacteria suggesting that it contains bioactive compounds that can serve as antibacterial agent(s) or lead compound for the synthesis of such agent(s). Although the antibacterial property of the leaf extract is related to its phytoconstituents especially alkaloids and also others, some alkaloids specifically those belonging to beta-carboline group possess antimicrobial, anti-HIV and antiparasitic activities (18). The potency of *A. macrophylla* against tested bacteria gives a piece of evidence for the medicinal value and provides the basis for its traditional usage as a remedy for skin infections like boils.

Acute toxicity study enables the researcher to determine the nature and degree of toxic reactions that may follow a single, usually large dose exposure to an agent, as may occur clinically in overdose. In this study, there was no evidence of toxicity in rats exposed singly to different doses of the leaf extract even when a dose of 5000 mg/kg body weight was used. Hence the ethanol leaf extract can be considered as being non-toxic following acute

administration. This observation of a good safety profile may explain why the plant's leaf is widely used in folkloric medicine.

Sub-acute toxicity studies on the other hand help identify any organ targeted toxic effects. The tests also help one to know the extent of non-observable adverse effect (19) as well as appropriate dose regimens for long-term studies. Alterations in body and organ weights of animals exposed to xenobiotics are accepted indicators of changes induced by the agents (20). In toxicological evaluation, the weights of vital organs are sensitive indicator of the effects of a xenobiotic for it defines toxicity as significant changes are shown in those organs (21). In this work, there was insignificant changes in the relative vital organs and lack of gross abnormalities signifying that the extract did not alter normal growth.

Hematopoiesis, a process that is vital to life can be affected by both conventional and herbal medications. Evaluation of hematological indices in animal models provides information on blood related benefits of plant extracts and also their toxic manifestations (22). Furthermore, the analysis is useful in risk assessment in humans as data from hematological changes in animals when translated have a marked predictive value for human toxicity. The results of hematological assessment in this study did not show any significant changes in hematological indices. The erythrocyte indices being a part of the above mentioned was not affected. It can be said that the leaf extract did not affect erythropoiesis, morphology or osmotic fragility of the red blood cells (23). The lack of significant alterations extended to the total white blood cells and its differentials. Since these cells are the major

effectors of both innate and adaptive immunities (24), it can be interpreted that the leaf extract has no effect on immune responses. Platelets counts were also not altered in this study, implying that the leaf extract has no effect on hemostasis, which is being controlled by platelets. The lack of alterations in hematopoietic indices may be a sign that the extract does not affect the bone marrow adversely since all the blood cells originate from the hematopoietic stem cells in the bone marrow (25). On the other hand, the observed effects may occur because the extract did not alter the main stimulants in the pathway namely, erythropoietin for erythropoiesis and thrombopoietin for thrombocytosis. All observations point to the non-hematotoxic nature of the extract at the doses used in this study. Other researchers working on varied plant extracts also recorded similar effects (26, 27, 28, 29). However, since changes in hematological parameters occur relatively slowly, the experimental duration may not have been enough to identify any alterations, and such was the findings with *Lychnophora trichocarpha* (30). Even though some of the phytochemicals found in the extract have established stimulant effect on hematopoiesis, the doses used may have been too small to allow such effects.

The serum chemistry evaluation was to assess the effect of the extract on hepatic and renal functions of treated rats compared to controls. The liver and kidney are important elimination and synthetic organs and analysis of their functions is extremely essential in toxic evaluation of xenobiotics (31). The ethanol leaf extract of *A. macrophylla* did not significantly alter any of the liver and kidney parameters assessed. A rise in serum levels of AST, ALT and ALP serve as an indicator of hepatotoxicity or liver diseases (32). Because serum proteins especially albumin are synthesized within the hepatocytes, their levels are used to assess liver's synthetic capacity (33). The absence of significant alterations of liver indices in extract-treated rats compared to control in this study suggests that the extract did not affect the hepatocytes. Many markers are used to evaluate kidney functions including serum creatinine which is the most sensitive and serum potassium, the most convincing electrolyte marker (34, 35). In this study, none of these markers was altered signifying that the extract is not nephrotoxic. Equally, there were no changes in the lipid profile of extract-treated rats compared to control. This implies that the extract did not affect the processes that may lead to increase levels of plasma lipoproteins especially low-density lipoproteins (LDL), which include hepatic cholesterol biosynthesis, increased fecal bile acid secretion and stimulation of receptor-mediated catabolism of LDL-cholesterol and uptake of LDL from blood in the liver (36).

Conclusion

The observations in this study suggest that the ethanol leaf extract of *A. macrophylla* has a broad spectrum

of antibacterial activities that can be exploited in the management of human infections due to the investigated bacteria. Findings also indicate that oral administration of the ethanol leaf extract is relatively safe as reflected in the results of acute and sub-acute studies. The observed antimicrobial potential and nontoxic effects of *A. macrophylla* ethanol leaf extract make it a candidate for bioassay guided and isolation of compounds which can possibly be developed into new lead structures for drug development programs against diseases.

Authors' contributions

GAE conceived the research idea. MUA designed the work. GCA wrote the first draft of the manuscript. GAA and AUM carried out the literature search. ADOJ and CCO carried out the statistical analysis. All authors read and approved the final manuscript.

Conflict of interests

The authors declare no conflict of interests.

Ethical considerations

The protocol for this study was confirmed by ethical committee (UNICAL/010/PA/2016) and the authors of this manuscript observed ethical issues. Animals were handled according to the International Guidelines for Care and Handling of Experimental Animals.

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