

Comparative Antibacterial Study of Aqueous and Ethanolic Leaf Extracts of *Annona Muricata*

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Abstract

This research project was aimed at investigating the antibacterial efficacy of aqueous and ethanol leaf extracts of *A. muricata* using agar-disc diffusion method for five clinical isolates of bacteria consisting of two gram-positive (*Staphylococcus aureus* and *Streptococcus mutans*) and three gram-negative (*Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhi*) bacteria which have been implicated in the most common types of bacterial infections such as diarrhea, typhoid fever, tooth decay, etc. The antibacterial activity was measured by the diameter of zone of inhibition (mm) observed and the extracts were found to exhibit antibacterial activities against the test organisms with each organism showing different patterns of formation of zones of inhibition at different concentrations of the extracts. The results of this study revealed that *S. aureus* was the most susceptible gram-positive bacteria and *S. typhi* was the most susceptible gram-negative bacteria which indicated that this plant contains compounds with wide antibacterial activity which validates their use for treatment of various microbial infections in traditional medicine. The findings in this study provide the basis for further study on the plant with the aim of isolating and identifying the active substances responsible for its antimicrobial activities. The plant could also be standardized to develop cheap, safer, culturally acceptable herbal medicines to help combat the problem of antibiotic resistance. The extraction of *A. muricata* which was done by maceration yielded 14.95% and 17.63% for aqueous and ethanol extracts respectively. The difference between both extracts when compared to the standard antibiotic (Ciprofloxacin) was significant ($p < 0.05$ level of significance) against *P. aeruginosa*, *E. coli* and *S. mutans*. Conversely, *S. aureus* and *S. typhi* showed no significant difference ($p < 0.05$ level of significance) in their response to both extracts (400 mg/ml) and the standard drug. This shows that *A. muricata* extract can be potential antimicrobial agent directed against *S. aureus* and *S. typhi*.

Keywords: *Annona muricata*, antibacterial, extracts, comparative, ethno-medicinal.

INTRODUCTION

The genus name *Annona* is from the latin word ‘anon’ meaning yearly produced referring to the fruit production habits of the various species in this genus. *Annona muricata* which is also known as ‘graviola’ or ‘soursoup’, is an ethno-medicinally important species from the Annonaceae family. It is adaptable to tropical climate and currently cultivated for its fruit in most South-East Asian countries such as Malaysia, Indonesia and the Philippines.

Tea preparation from the leaf of *A. muricata*, is used as a sedative in the West Indies and Peruvian Andes. This infusion is also used to relief pains or for antispasmodic purposes. The various species of the Annonaceae family includes: *Annona squamosa*, *Annona reticulata*, *Annona montana*, *Annona glabra*, *Annona clossiflora*, *Annona cherimola*, *Annona sylvatica*, *Annona muricata*, just to mention but a few. It has a long, rich history of use in herbal medicine as well as a lengthy recorded indigenous use. In the Peruvian Andes, a leave tea is used for catarrh (inflammation of mucus membranes). In the Peruvian Amazon, the bark, roots and leaves are used for diabetes and as a sedative. In the Brazilian amazon, a leave tea is used for liver problems, rheumatism and arthritis pain. In Jamaica, Haiti and the West Indies, the fruit and/or juice is used for fevers, parasites and diarrhea. The bark or leaf is used for heart conditions, coughs, difficult childbirth, flu, asthma, hypertension and parasites

A. muricata crushed leaves are applied to mature boils and abscesses, used as remedy for distention and dyspepsia, scabies, and skin diseases. Powder of dried leaves and sap from fresh plant parts are useful in destroying vermin. All the tree parts have insecticidal properties and can be used with fruit as bait for fishing. The green bark is rubbed on wounds to stop bleeding (Orwa *et al.*, 2009).

Antimicrobial agents are substances that kill microorganisms or inhibit their growth. They are widely employed to cure bacterial diseases. Antimicrobial agents that reversibly inhibit growth of bacteria are called bacteriostatic whereas those with irreversible lethal action on bacteria are known as bactericidal (Rajesh and Rattan, 2008). Ideally, antimicrobial agents disrupt microbial processes or structures that differ from those of the host. They may damage pathogens by hampering cell wall synthesis, inhibiting microbial protein and nucleic acid synthesis, disrupting microbial membrane structure and function, or blocking metabolic pathways through inhibition of key enzymes (Willey *et al.*, 2008).

Before an antimicrobial agent is accepted for use in human beings, it must demonstrate most, if not all of the following properties: selective toxicity (it should act on bacteria without damaging host tissues); bactericidal

rather than bacteriostatic; effective against a broad range of bacteria; non – allergic; active in plasma, body fluids, etc. It should also be stable, preferably water soluble; desired levels should be reached rapidly and maintained for adequate period of time; it should not give rise to resistance in bacteria; it should have long shelf life; and, it should not be expensive (Rajesh and Rattan, 2008).

The effectiveness of chemotherapeutic agents depends on many factors, some of which include; the route of administration and location of the infection, the presence of interfering substances, the concentration of the drug in the body, the nature of the pathogen, the presence of drug allergy, and another factor that should not be overlooked is the resistance of microorganisms to the drug.

A great number of antibacterial agents exist for various purposes; some of these are usually in the form of plants. The action of these plants on microorganisms have been found to be due to the presence of certain substances such as alkaloids, glycosides, volatile oils, gums, tannins, steroids, saponins, phlobatannins, flavonoids, and a host of other chemical compounds referred to as secondary metabolites that are present in them (Sofowora, 1993; Oyagade *et al.*, 1999). Medicinal plants have played a major role in the treatment of various diseases including bacterial and fungal infections.

Traditional system of medicine which depends mainly on medicinal plants is rich in ethnomedical knowledge of the uses of medicinal plants in the treatment of infectious conditions (Iwu, 1993). These medicinal plants that are employed in traditional medicine, represents potential sources of cheap and effective standardized herbal medicines (phytomedicine) and leads in the discovery of novel molecules for the development of new chemotherapeutic agents (Farnsworth and Morris, 1976). Several infectious diseases including malaria, diarrhea, dysentery, gonorrhoea and fungal infections have been successfully managed in traditional medical practice employing medicinal plants (Sofowora, 1993).

The antibacterial effect of the ethanolic and aqueous extracts of the leaves of *Annona muricata* were tested against various bacterial strains such as *Staphylococcus aureus* ATCC29213, *Escherichia coli* ATCC8739, *Proteus vulgaris* ATCC13315, *Streptococcus pyogenes* ATCC8668, *Bacillus subtilis* ATCC12432, *Salmonella typhimurium* ATCC23564, *Klebsiella pneumoniae* NCIM No. 2719 and *Enterobacter aerogenes* NCIM No. 2340.

Olawale *et al.*, studied the anti-hyperglycemic activity of the methanolic extract of *A. muricata* on streptozotocin-induced diabetic Wistar rats. A mean blood glucose concentration of 3.78 ± 0.190 mmol/L, 21.64 ± 2.229 mmol/L and 4.22 ± 0.151 mmol/L for the control, untreated diabetic and treated diabetic groups respectively were selected. A significant difference in the blood glucose concentrations of the treated and untreated hyperglycemic groups of rats was observed (Adeyemi *et al.*, 2009).

A. muricata may have anti-depressive activity due to its ability to stimulate serotonin receptors. The fruit and the leaf extracts of Guyabano contains three alkaloids, annonaine, nornuciferine and asimilobine, that upon tests have shown to inhibit binding of [³H] rauwolscine to 5-HT_{1A} receptors in calf hippocampus. These results imply that Guyabano fruit (*A. muricata*) possesses anti-depressive effects (Hasrat *et al.*, 1997).

MATERIALS AND METHODS

Fresh leaves of *A. muricata* were collected from a private garden in Barnawa, Kaduna South Local Government area of Kaduna State Nigeria. The plant was taxonomically identified and authenticated in the herbarium of biological sciences department of Ahmadu Bello University Zaria and given the voucher number 70707. The leaves were washed properly with clean water and air dried at room temperature for several days until well dried, after which it was grounded to fine powder using laboratory mortar and pestle and stored in an air-tight container for further use.

Test organisms

Clinical isolates of five bacteria strains were selected and used in this study based on their availability and pathogenicity. Two Gram- positive and three Gram – negative bacteria were selected and isolates were collected from Barau Dikko Specialist hospital, Gwamna Awan hospital and NNPC clinic all in Kaduna state Nigeria. The selected organisms include: *Staphylococcus aureus*, *Streptococcus mutans*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhi*. Freshly prepared subcultures were used for this study.

Preparation of stock solution of extracts

5g of each of the extracts (aqueous and ethanolic) was weighed separately and transferred into two reagent bottles, 10ml of dimethyl sulfoxide was added and mixed properly until a homogenous solution was obtained. This served as the stock solution of 500mg/ml for each extract. From the stock, various concentrations of 400mg/ml, 200mg/ml, 100mg/ml and 50mg/ml were prepared. The various concentrations of the extracts was poured into endpolf tubes and stored for further use Hasrat *et al.*(1997)

Preparation of extract impregnated paper discs.

The extract impregnated paper discs were prepared as described by Ekundayo and Ezeogu (2006). Whatman No.

1 filter paper was cut into discs of 7mm diameter using an office perforator. The discs were placed in sterile bujou bottles and sterilized in hot air oven at 160°C for an hour. About 25 discs were placed in each bujou bottle and the various extracts were added to each tube to soak the discs, the soaked discs were then dried in an oven until it was totally dried. Discs of dimethyl sulfoxide, water and ciprofloxacin which were used as controls were also prepared in like manner.

Ciprofloxacin was used as the positive control (standard drug) due to its broad- spectrum antibacterial activity for both Gram- positive and Gram- negative bacterial strains, while dimethyl sulfoxide and water were used as negative control because they were used as solvent for dissolving the extracts and drug respectively.

Extraction

The extraction was done by maceration using ethanol and distilled water as solvents. 100g of the dried powder of *Annona muricata* leaves was weighed into two separate beakers and 1000ml of the solvents were added to each of the beakers containing the powder, the mixture was allowed to stand for 3days at room temperature with agitations at intervals (Adamu *et al.*; 2012). The extracts were filtered separately through a muslin cloth and then through Whatman No. 1 filter paper. The extract was concentrated to dryness using a rotary evaporator to remove the solvents.

Gram staining

A smear of the various cultures was made on clean glass slides. The smears were stained with crystal violet solution for 60 seconds (primary dye) and then washed with tap water and drained off. The slide was flooded with iodine solution for 30 seconds and washed again with tap water. The glass slide was tilted and alcohol (95% methanol) was added in drops to decolorize until all the blue colour of the stain was removed. It was then washed with tap water. The slide was flooded with carbol- fuchsin (counter stain) for 60 seconds. All the slides were examined under oil immersion(Oyeleke and Manga 2008).

Catalase test

A drop of hydrogen peroxide (3%) was placed on a clean glass slide, a bit of growth from a solid medium was introduced to the hydrogen peroxide in the slide using a wire loop. A positive test was indicated by bubbling and frothing while in a negative test there was no bubbling and frothing .(Oyeleke and Manga 2008)

Indole test

Each organism was grown in 5ml of peptone water and incubated for 24hrs after which 3-8 drops of Kovacs indole reagent was added and shaken gently. A positive test was indicated by development of a red colour in the reagent layer above the broth within 1 minute. In a negative test, the indole reagent retains its yellow colour .(Oyagade *et al* 1997)

Coagulase test (slide method)

Two drops of normal saline were placed 2cm apart on a clean, grease free glass slide that has been divided into two with a grease pencil. Colonies from each culture were carefully emulsified in each drop of saline. A loopful of citrated human plasma was added to the bacterial suspension on one side and mixed with wire loop, the slide was held up and tilted back and forth for 1 minute. Clumping of cells in the bacterial suspension mixed with plasma indicates a positive coagulase test while in a negative test there is no clumping of cells .

Test for Antibacterial Activity

The antibacterial test of *Annona muricata* leaf extracts was done using agar- disc diffusion method described by Adamu *et al*, 2012. In this case, freshly prepared media was used. Freshly prepared media was poured into sterile petri- dishes and allowed to solidify. Pure culture of the test organisms were inoculated aseptically using sterile wire- loop unto the surface of the media until evenly distributed. The plates were labeled appropriately and discs impregnated with extracts and controls were placed unto the inoculated media and covered. The petri- dishes were incubated at 37°C for 24- 48hrs. The diameter of the zone of inhibition formed was measured using a transparent plastic ruler (Ekundayo *et al*, 2006) and the tests were carried out in triplicate and the mean values were recorded.

RESULTS AND DISCUSSIONS

The extraction carried out in this study was by maceration using ethanol and water as solvents and the percentage yield for both extracts were calculated with aqueous and ethanolic extracts having a yield of 14.95% and 17.63% respectively.

Table 1: Result of biochemical test carried out to confirm test organisms.

Test organisms	Gram staining	Coagulase test	Indole test	Catalase test
<i>Salmonella typhi</i>	-	-	-	+
<i>Staphylococcus aureus</i>	+	+	+	+
<i>Streptococcus mutans</i>	+	-	-	-
<i>Pseudomonas aeruginosa</i>	-	-	-	+
<i>Escherichia coli</i>	-	+	+	+

The + sign indicates a positive test while the - sign indicates a negative test. Gram staining was used to differentiate Gram positive from Gram negative bacteria, coagulase test was used to identify *Staph aureus* from other coagulase negative *Staphylococcus spp.* Indole test was used to identify organisms that have the enzyme tryptophanase which converts tryptophan to pyruvic acid, indole and ammonia, while catalase test identifies organisms that have catalase enzyme which breaks down hydrogen peroxide to H₂O and O₂.

Table 2: Result of antibacterial activity of aqueous extract of *A. muricata* and control measured as zone of inhibition diameter (mm) at various concentrations.

Organisms	Extract concentrations (mg/ml)				Control (100mg/ml)
	50	100	200	400	Ciprofloxacin
<i>Escherichia coli</i>	3.50±6.0 ^a	5.83±5.11 ^a	6.17±5.35 ^a	6.50±5.63 ^a	48.50 ^b
<i>Pseudomonas aeruginosa</i>	0.00	3.17±5.48 ^a	7.33±6.43 ^a	14.00 ±4.62 ^a	41.50 ^b
<i>Streptococcus mutans</i>	3.17±5.48 ^a	3.50±6.06 ^a	4.17 ±7.22 ^a	14.00 ±3.97 ^a	53.50 ^b
<i>Staphylococcus aureus</i>	3.33±5.77 ^a	4.00±6.93 ^a	11.00±1.73 ^a	34.17±42.72 ^a	42.50 ^a
<i>Salmonella typhi</i>	3.00±5.20 ^a	3.33±5.77 ^a	3.30±5.77 ^a	22.33±19.44 ^a	44.00 ^a

Values are given as mean ± standard deviation (SD), n= 3. values with superscript a across the row show no significant difference at (p< 0.05) while those with superscript b show significant difference with the rest in the group across the row.

Table 3: Result of Antibacterial Activity of Ethanolic Extract of *Annona muricata* and Control Measured as Zone of Inhibition Diameter (mm) at Various Concentrations.

Organisms	Extract concentrations (mg/ml)				Control (100mg/ml)
	50	100	200	400	Ciprofloxacin
<i>Escherichia coli</i>	2.67±4.62 ^a	3.30±5.77 ^a	7.33±6.35 ^a	10.17±0.29 ^a	48.50 ^b
<i>Pseudomonas aeruginosa</i>	0.00	2.83±4.90 ^a	6.17±5.35 ^a	7.00±6.08 ^a	41.50 ^b
<i>Streptococcus mutans</i>	0.00	9.33±0.76 ^a	11.30±3.6 ^a	14.33±3.26 ^a	53.50 ^b
<i>Staphylococcus aureus</i>	0.00	3.00±5.20 ^a	3.00±5.20 ^a	16.67±12.85 ^a	42.50 ^a
<i>Salmonella typhi</i>	3.17±5.48 ^a	3.17±5.48 ^a	6.17±5.35 ^a	32.17±1.26 ^a	44.00 ^a

Values are given as mean ± standard deviation (SD), n= 3. values with superscript a across the row show no significant difference at (p< 0.05) while those with superscript b show significant difference with the rest in the group across the row.

DISCUSSION

Extraction of *A. muricata* which was carried out by maceration according to the method described by Adamu *et al.*, 2012 gave a percentage yield of 14.95% and 17.63% for aqueous and ethanol extracts respectively. This result shows that the ethanol extract had more yield than the aqueous extract which could be due to ethanol's ability to dissolve more of the active components of the plant than water.

The clinical isolates of the organisms collected from various government hospitals in Kaduna were subjected to various biochemical tests to confirm their identity and also differentiate Gram- positive from Gram-negative bacteria as shown in using the method described by Oyeleke *et al.*, 2008. The test organisms showed different pattern of zone of inhibition for each extract and the antibacterial activity of extracts increased with increase in concentration which is in line with other studies by Adamu *et al.*, 2012.

From table 2. The aqueous and ethanolic extracts of *A. muricata* all showed antibacterial activity against the test organisms and the organisms showed different response for each extract as revealed by the observed zone of inhibition. The antibacterial effect was shown to be concentration-dependent.

According to Sofowora (1973) *A. muricata* extracts were found to have various secondary metabolites (phytochemicals) such as flavonoids, tannins, acetogenins, saponins, alkaloids, glycosides, steroids, phlobatannins, volatile oils, etc which are said to be responsible for the action of plants on micro organisms.

Staphylococcus aureus, a gram-positive bacteria had the highest zone of inhibition for both extracts with the average values of 34.17mm and 16.67mm at 400mg/ml for aqueous and ethanolic extracts respectively which is consistent with the report of Vijayameena *et al.*, 2009. While *Salmonella typhi*, a Gram-negative bacteria had the highest zone of inhibition as well with average values of 22.33mm and 32.17mm for aqueous and ethanolic extracts respectively. From this result, it was observed that *S. aureus* showed more sensitivity to the aqueous extract with zone of inhibition of 34.17mm for aqueous extract against 16.67mm for ethanolic extract. On the other hand, *S. typhi* showed more sensitivity to the ethanolic than aqueous extract with zones of inhibition of 22.33mm and 32.17mm for aqueous and ethanolic extracts respectively.

Discs of dimethyl sulfoxide and distilled water which were used as negative control did not show any zone of inhibition. Their use as negative control is because they were used as solvents to dissolve the extracts and ciprofloxacin respectively. This result suggests that the antibacterial activity observed was strictly due to the extract and ciprofloxacin not the solvents.

Statistical analysis (Student t-test, independent sample) was carried out on the data obtained from this research work using Statistical Package for Social Sciences (SPSS) version 16.

The Statistical analysis at ($P < 0.05$ level of significance) revealed that there is no significant difference between the observed antibacterial activity of aqueous and ethanolic extracts of *A. muricata* at all concentrations used which suggests that the active component responsible for the antibacterial activity of the extracts by the two solvents are similar. Even though there was more yield of the ethanolic extract, its antibacterial activity when compared to the aqueous extract which had lower yield showed no significant difference. This could mean that active components other than those responsible for the antibacterial activity of the plant were dissolved by ethanol thereby resulting in the higher yield observed for the ethanolic extract.

The difference between both extracts when compared to the standard antibiotic (ciprofloxacin) was significant ($P < 0.05$ level of significance) against *P. aeruginosa*, *E. coli* and *S. mutans*. Conversely, *S. aureus* and *S. typhi* showed no significant difference ($P < 0.05$ level of significance) in their response to both extracts (400mg/ml) and the standard drug. This shows that *A. muricata* extract can be a potential antimicrobial agent directed against *S. aureus* and *S. typhi*.

CONCLUSION

The result of this work shows that both aqueous and ethanolic extracts of *A. muricata* have antibacterial activity against all the test organisms with *S. aureus* being the most susceptible Gram-positive bacteria and *S. typhi* as the most susceptible Gram-negative bacteria. Also this further potentiates the use of plants as a base for the development of a medicine (a natural blueprint for new drug development) and also as a phytomedicine used for the treatment of diseases. Iwu (1993).

Also, this study shows that *A. muricata* can be exploited for the development of new potent antibiotics which will help combat the problem of antibiotic-resistance.

When properly formulated, *A. muricata* can be used for the treatment of bacterial infections caused by the test organisms such as, urinary tract infection, neonatal meningitis, respiratory tract infection, enteric (typhoid) fever, dermatitis, etc caused by *E. coli*, *S. aureus*, *S. typhi*, and *P. aeruginosa* respectively.

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