*Lampiran 1* (Literatur Ke-1)

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# EFEK EKSTRAK METANOL DAUN SIRSAK *(Annona muricata)* DALAM MENGHAMBAT PERTUMBUHAN BAKTERI *Salmonella typhi* SECARA IN VITRO

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## ABSTRAK

*Salmonella typhi* merupakan penyebab utama penyakit demam typhoid. Masalah yang kini dihadapi dari berbagai penyakit infeksi adalah resistensi bakteri terhadap antibiotik. Pengembangan suatu alternatif pengobatan yang tidak menyebabkan efek samping perlu dilakukan. Salah satu tanaman yang telah lama dimanfaatkan sebagai obat tradisional adalah daun sirsak. Kandungan flavonoid, steroid, alkaloid, saponin dan tanin yang dilaporkan pada daun tanaman sirsak *(Annona muricata)* berkontribusi sebagai antibakteri. Penelitian ini bertujuan untuk mengetahui efek antibakteri dengan mengukur zona hambat yang diinduksi oleh ekstrak methanol daun sirsak pada bakteri *Salmonella typhi* secara *in vitro*. Penelitian ini merupakan penelitian eksperimental laboratorium dengan rancangan penelitian *posttest only control design* dengan metode difusi agar *(Kirby-Bauer).* Studi ini menggunakan daun sirsak yang diekstraksi dengan metode maserasi dengan menggunakan pelarut metanol. Ekstrak metanol daun sirsak dengan konsentrasi larutan 50mg/mL, 100mg/mL, 200mg/mL dan 400mg/mL yang diuji pada bakteri *S. typhi* secara *in vitro*. Uji ekstrak secara *in vitro* pada bakteri
*S. typhi* memperoleh hasil, ekstrak metanol daun sirsak tidak dapat menghambat pertumbuhan bakteri
*S. typhi* secara *in vitro*. Terdapat beberapa faktor yang tidak dapat dikontrol yang mempengaruhi hasil dari penelitian ini seperti, jenis bakteri, lingkungan tempat tanaman berasal dan efektifitas ekstrak. Diperlukan penelitian lebih lanjut untuk uji antibakteri ekstrak methanol daun sirsak dengan konsentrasi larutan yang berbeda, untuk mengetahui konsentrasi yang dapat memberikan efek secara *in vitro* pada bakteri *S. typhi.*

**Kata kunci:** *Salmonella typhi,* ekstrak daun sirsak, antibakteri

## ABSTRACT

*Salmonella typhi (S. typhi)* still the main cause of typhoid fever. The new problem of various infectious diseases are the bacterial resistance towards antibiotic. The development of other alternative treatment which would not making any side effect for patient is a necessary. One of the plant that has been used as traditional medicine is soursop leaves *(Annona muricata).* Soursop leaves have kinds of natural materials which contain tannins, alkaloids, saponins and flavonoids, which is reported has function as an antibacterial. This study was aimed to determine the antibacterial effect by measured the inhibition zone of soursop leaves extract on growth of *in vitro S. typhi.* This research conducted as an experimental laboratory using research design posttest only control design with Kirby-Bauer modification method.

The subject of this study are soursop leaves which were extracted by maceration method using methanol. The methanol extract of soursop leaves were tested with various concentration, range from 50mg/mL, 100mg/mL, 200mg/mL and 400mg/mL. Extraction result of soursoup leaves were tested toward *in vitro S. typhi* showed that the soursop leaves extracts did not have inhibition effect against *S. typhi*. There are some factors that affect the result such as the type of the bacteria, the environment of plants origin and the effectiveness of the extract it self. It is necessary to do further research on antibacterial effect from methanol extract of soursop leaves with another concentration which can give antibacterial effect towards *S. typhi*.

**Keywords:** *Salmonella typhi*, soursop leaves extract, antibacteria

## PENDAHULUAN

Demam tifus merupakan penyakit infeksi yang disebabkan oleh bakteri gram negatif *Salmonella typhi (S. typhi).* Tingginya angka morbiditas dan mortalitas karena demam tifus terutama pada negara berkembang, menggerakkan berbagai pihak untuk berupaya mencari modalitas baru antibiotik sebagai solusi menangani masalah demam tifus. Berkembangnya paradigma dalam bidang kesehatan, untuk menggunakan ramuan alami dan obat-obat tradisional sebagai terapi penyakit, sangat membuka peluang untuk meneliti dan mengembangkan tanaman obat sebagai cadangan antibiotik di masa depan.[1,2]

Salah satu tanaman yang telah lama dimanfaatkan sebagai obat tradisional adalah daun sirsak *(Annona muricata)*. Kandungan flavonoid, steroid, alkaloid, saponin dan tanin yang dilaporkan pada daun tanaman sirsak berkontribusi sebagai antibakteri. Penelitian terdahulu telah melakukan uji daya hambat ekstrak metanol daun sirsak pada berbagai jenis bakteri gram negatif dan gram positif. Namun belum ada data ilmiah terkait penelitian ekstrak daun sirsak pada bakteri gram negatif *S. typhi.* Sehingga penelitian uji daya hambat ekstrak daun sirsak dalam menghambat pertumbuhan bakteri *S. typhi* secara *in vitro* perlu untuk dilakukan agar nantinya dapat dijadikan sebagai dasar penelitian selanjutnya.[1,2,3]

## BAHAN DAN METODE

Penelitian ini dilakukan di Laboratorium Mikrobiologi Fakultas Kedokteran Universitas Udayana pada bulan Maret 2016 – Januari 2017. Jenis penelitian ini adalah penelitian *True Experimental Posttest only Control Group*, sehingga dapat mengetahui aktivitas antibakteri dari ekstrak metanol daun sirsak dalam menghambat pertumbuhan *S.typhi* yang dinilai berdasarkan parameter diameter zona hambat. Pengujian dilakukan dengan metode difusi agar *(Kirby-Bauer)*, dengan menggunakan empat konsentrasi ekstrak, yakni 50mg/mL(P1), 100mg/mL(P2), 200mg/mL(P3) dan 400mg/mL(P4), serta menggunakan pembanding kontrol negatif methanol (K1) dan kontrol positif ciprofloxacin (K2). Daun sirsak yang digunakan, diperoleh dari tanaman sirsak yang tumbuh di Kabupaten Klungkung, Bali, dengan memilih daun yang berwarna hijau, sudah dewasa, dan tidak terserang hama penyakit.

Daun sirsak yang sudah dibersihkan dengan air kemudian dipotong-potong kecil menggunakan pisau, kemudian dikering-anginkan tanpa sinar matahari atau dengan menggunakan oven dengan suhu $40^0$C. Setelah kering, daun sirsak dihaluskan dengan blender dan diayak untuk memisahkan bagian yang masih kasar dengan yang sudah halus. Setelah itu, 500 gram serbuk simplisia daun sirsak direndam dengan larutan metanol sebanyak 2 liter di dalam sebuah toples kaca, lalu ditutup. Biarkan selama 2 hari sambil sesekali diaduk. Setelah 2 hari, rendaman tersebut disaring dengan kertas saring sehingga menghasilkan filtrat 1 dan ampas 1. Setelah itu filtrat (lapisan atas yang berupa campuran metanol dengan zat aktif) diambil dan residu dimaserasi kembali selama semalam. Selanjutnya filtrat diambil lagi dan residu dimaserasi kembali sampai empat kali pengulangan. Setelah semua filtrat ekstrak daun sirsak diperoleh, lalu difiltrasi menggunakan kertas *Whatmann 0,1* dan dievaporasi menggunakan *rotary evaporator* pada suhu $40^0$C sehingga diperoleh ekstrak kental. Ekstrak yang telah ditimbang dan disimpan dalam wadah gelas atau botol yang tertutup dengan berat 0.05 gr, 0.1 gr, 0.2 gr, dan 0.4 gr dan sudah diberi label pada botol, diencerkan atau dilarutkan dengan menambahkan larutan metanol 1000 cc pada masing- masing botol, kemudian diaduk hingga ekstrak larut dalam botol.

Media yang digunakan untuk menumbuhkan bakteri *S. typhi* adalah media agar *Mueller-Hinton* (MH). Bakteri *S. typhi* yang dipakai didapat dari Laboratorium Mikrobiologi FK UNUD yang direjuvenasi terlebih dahulu sebelum digunakan. Biakan bakteri yang berumur 24 jam disuspensi sehingga kekeruhannya setara 0,5 *Mc*

*Farland* dengan konsentrasi bakteri 1 x $10^8$ CFU/ml. Suspensi bakteri kemudian dioleskan ke media agar kemudian diletakkan 6 disk yang masing-masing berisi empat macam konsentrasi ekstrak, kontrol negative dan kontrol positif. Jumlah pengulangan yang dilakukan dalam penelitian ini adalah sebanyak 4 kali sesuai perhitungan rumus *Federer*. Lempeng agar yang sudah berisi bakteri *S.typhi*, bahan uji, dan control diinkubasi selama 18-24 jam pada suhu 37$^o$
C. Setelah inkubasi, data diambil dan dikumpulkan adalah data kuantitatif berupa diameter zona hambat yang diukur menggunakan jangka sorong, kemudian dilakukan analisis data.

Adapun tahapan yang diambil dalam penelitian ini diawali dengan pembuatan proposal, meminta izin dari komite etik, meminta izin penggunaan Laboratorium Mikrobiologi FK Udayana, pencarian bahan ekstrak dan persiapan isolat bakteri. Tahap pelaksanaan penelitian berupapembuatan ekstrak, persiapan bakteri, uji *in vitro* dan pengukuran hasil berupa data kuantitatif. Penelitian

| Golongan senyawa | Metode pengujian | Pengamatan | Hasil |
|---|---|---|---|
| | Meyer | Tidak terbentuk endapan putih | – |
| Alkaloid | Bouchar-dat | Terbentuk endapan hitam | + |
| | Wagner | Terbentuk endapan coklat | + |
| Saponin | Foam | Terbentuk busa yang Stabil | + |
| Flavonoid | Pew | Terbentuk warna kuning Intensif | + |
| Steroid | Lieberm an-Burchard | Terbentuk cincin warna biru kehijauan | + |
| Triterpenoid | Lieberm an-Burchard | Tidak terbentuk cincin kecoklatan | – |
| Fenol | $FeCl_3$10 % | Terbentuk warna biru kehitaman | + |
| Glikosida | Lieberm an-Burchard | Terbentuk warna Hijau Kebiruan | + |
| Tanin | Pbasetat 10% | Terbentuk endapan putih | + |

ini sudah mendapatkan izin dari Komisi Etik Fakultas Kedokteran Universitas Udayana dengan nomor surat 362/UN.14.2/KEP/2016.

**HASIL**

Uji fitokimia dilakukan untuk mengetahui jenis senyawa metabolit sekunder yang terdapat didalam ekstrak metanol daun sirsak. Uji fitokimia dilakukan di Laboratorium Fitokimia Farmasi Universitas Udayana.

**Tabel 1.** Hasil uji fitokimia ekstrak metanol daun sirsak (*Annona muricata)*

Pengukuran diameter zona hambat dilakukan setelah lempeng agar dengan bakteri *S. Typhi* diinkubasi di dalam inkubator selama 18-24 jam dengan suhu $37^0$C. Diameter zona hambat diukur menggunakan jangka sorong dengan ketelitian 0,05 mm pada zona bening di sekitar cawan petri.

**Gambar 1.** Hasil uji ekstrak metanol daun sirsak terhadap pertumbuhan bakteri *S. Typhi* secara *in vitro* pada pengulangan pertama.

Dari zona hambat yang ditunjukan pada gambar di atas, pengukuran dilakukan dari beberapa sisi lingkaran kemudian dirata-ratakan sehingga didapatkan hasil rerata K1 = 0,00 mm, K2 = 32,25 mm, P1 = 0,00 mm, P2 = 0,00 mm, P3 = 0,00 mm, P4 = 0,00 mm. Hasil dapat dilihat pada tabel 2.

**Tabel 2.** Data hasil pengukuran diameter zona hambat ekstrak metanol daun sirsak terhadap pertumbuhan bakteri *S. typhi* secara *in vitro*

| Pengulangan | K1 mm | K2 mm | P1 mm | P2 mm | P3 mm | P4 Mm |
|---|---|---|---|---|---|---|
| I | 0,00 | 35,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| II | 0,00 | 31,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| III | 0,00 | 32,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| IV | 0,00 | 31,00 | 0,00 | 0,00 | 0,00 | 0,00 |

**PEMBAHASAN**

Hasil pengukuran diameter zona hambat dengan metode *disk diffusion* menunjukan bahwa ekstrak metanol daun sirsak tidak memberikan efek antibakteri pada bakteri gram negatif *S. typhi* secara *in vitro.* Berbeda dengan hasil yang telah diperoleh oleh penelitian terdahulu, pengujian ekstrak metanol daun sirsak yang dilakukan pada kelompok bakteri gram negatif menunjukan adanya aktifitas anti bakteri seperti yang dilakukan oleh Ginda Haro, yang menguji pada bakteri *Escherichia coli***,** dimana pada konsentrasi minimalnya (5mg/mL) mampu menghambat pertumbuhan bakteri dengan zona inhibisi 8.0 mm. Dian Riani dkk, yang juga menguji ekstrak metanol daun sirsak pada bakteri gram negative *E. coli* memperoleh hasil bahwa ekstrak yang digunakan mampu menghambat pertumbuhan bakteri secara *in vitro* dengan diameter zona hambat 3.0 mm.[10,11]

Sebagian besar penelitian

pendahuluan yang menguji aktivitas antibakteri pada ekstrak daun sirsak, menggunakan bakteri gram negatif yang berasal dari spesies *E. coli*, maka dari itu dapat dipahami apabila hasil yang peneliti dapatkan berbeda dengan apa yang didapat oleh penelitian

sebelumnya. Perbedaan ini dapat terjadi mengingat spesies bakteri yang digunakan berasal dari spesies yang berbeda. Spesies bakteri yang berbeda akan menimbulkan mekanisme pertahanan yang berbeda terhadap antibakteri, baik dari komponen dinding sel bakteri maupun materi genetik yang dibawa.[10,11,12]

Perbedaan hasil uji penelitian ini dapat pula terjadi karena adanya kemungkinan resistensi bakteri yang digunakan sebagai sampel uji. Resistensi bakteri secara genetik terhadap zat tertentu perlu diperhitungkan mengingat sampel berasal dari isolat pasien Laboratorium Mikrobiologi RSUP Sanglah. Mekanisme strain bakteri yang mengalami resistensi terhadap antibiotik bisa diakibatkan dari pemakaian antibiotik dalam jangka waktu yang relatif lama dan terus-menerus, yang memungkinkan bakteri membentuk mekanisme pertahanan diri. Selain itu, faktor kepatuhan dari diri pasien, juga dapat memengaruhi terjadinya resistensi antibiotik jika pasien tidak memiliki kepatuhan dalam mengonsumsi antibiotik dengan benar.[12]

Hasil uji fitokimia yang dilakukan di Laboratorium Farmasi Universitas Udayana menyatakan bahwa ekstrak metanol daun sirsak mengandung metabolit sekunder yang bersifat antibakteri seperti flavonoid, steroid, alkaloid, saponin, dan tannin. Flavonoid dilaporkan memiliki mekanisme sebagai antibakteri. Adapun mekanisme reaksi antibakteri yang ditimbulkan oleh flavonoid seperti mampu menginhibisi sintesis asam nukleat bakteri, menginhibisi fungsi dari membran sitoplasmik, serta mampu menginhibisi metabolisme energi pada bakteri.[13] Steroid tersebar di alam sebagai fraksi lipid dari tanaman maupun hewan. Steroid dibentuk oleh bahan alam yang disebut sterol. Sterol merupakan senyawa yang terdapat pada lapisan malam (lilin) buah dan daun yang berfungsi sebagai pelindung diri dari serangan serangga dan serangan mikroba. Saponin berfungsi sebagai antibakteri dengan menyebabkan kebocoran protein dan enzim dari dalam sel. Selain itu saponin dapat menjadi antibakteri karena zat aktif permukaannya mirip detergen. Kemiripan sifat saponin dengan detergen akan menyebabkan penurunan tegangan permukaan dinding sel bakteri, merusak permeabilitas membrane dan akan sangat mengganggu kelangsungan hidup bakteri. Alkaloid sebagai antibakteri bekerja dengan cara mengganggu komponen penyusun peptidoglikan pada sel bakteri sehingga lapisan dinding sel tidak terbentuk secara utuh dan menyebabkan kematian sel. Selain itu, komponen alkaloid diketahui dapat berfungsi sebagai interkelator DNA dan menghambat enzim topoisomerase sel bakteri. Tanin sendiri merupakan senyawa yang bersifat lipofilik, sehingga mudah terikat pada dinding sel dan mengakibatkan kerusakan dinding sel bakteri.[11,13]

Hasil uji fitokimia yang dilakukan peneliti dengan uji fitokimia pada penelitian sebelumnya memang memiliki kesamaan, yang menyatakan bahwa ekstrak metanol daun sirsak memiliki potensi sebagai antibakteri apabila dilihat dari metabolit sekunder yang dikandungnya. Namun hasil uji *in vitro* yang peneliti lakukan pada bakteri jenis gram negatif dalam hal ini adalah bakteri *S. typhi* memberikan hasil bahwa potensi antibiotik yang dimiliki ekstrak metanol daun sirsak tidak memberikan efek antibiotik pada bakteri tersebut. Perbedaan hasil uji ini akan sangat tergantung pula pada efektifitas ekstrak yang digunakan.

Efektifitas ekstrak yang digunakan pada masing-masing penelitian sangat dipengaruhi oleh adanya variasi biologis dari tanaman yang digunakan. Tempat asal dari tanaman sirsak yang daunnya digunakan, diketahui dapat memengaruhi jumlah kandungan bahan aktif yang ada. Faktor-faktor lingkungan seperti suhu lingkungan, kelembaban relatif, radiasi matahari, angin, jenis unsur hara dalam tanah, ketersediaan air, ketercukupan cahaya dalam proses fotosintesis akan sangat memengaruhi fungsi fisiologis, bentuk anatomis dan siklus hidup tumbuhan. Faktor lingkungan inilah yang mungkin memengaruhi kadar senyawa metabolit sekunder yang dihasilkan oleh daun.[14]

Perbedaan hasil uji antibakteri antara peneliti satu dengan lainnya dapat pula dipengaruhi oleh ekstrak bahan alam yang digunakan. Tidak adanya standarisasi pembuatan ekstrak bahan alam antara laboratorium satu dengan yang lain akan menimbulkan hasil yang berbeda pula. Selain prosedur standar pembuatan ekstrak, faktor lain yang dapat memengaruhi mutu ekstrak yang digunakan adalah faktor kimia seperti jenis dan jumlah senyawa kimia serta metode ekstraksi dan pelarut yang digunakan.[14]

## SIMPULAN

Adapun simpulan dari penelitian ini adalah ekstrak metanol daun sirsak pada konsentrasi 50mg/mL, 100mg/mL, 200mg/mL dan 400mg/mL tidak dapat menghambat pertumbuhan bakteri *S. typhi* secara *in vitro*.

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*Lampiran 2* (Literatur Ke-2)

# 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.aeroginosa, 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, gonorrhea 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 pneumoneae* 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 + 2.229mmol/L and 4.22 + 0.151mmol/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-HTergic 5-HT1A 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 ependolf 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 $160^0$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 decolourize 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 $37^0$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 |
|---|---|---|---|---|
| ***Salmonella typhi*** | – | – | – | + |
| ***Staphylococcus aureus*** | + | + | + | + |
| ***Streptococcus mutans*** | + | – | – | – |
| ***Pseudomonas aeruginosa*** | – | – | – | + |
| ***Escherichia coli*** | – | + | + | + |

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 $H_20$ and $O_2$.

**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 |
| ***Escherichia coli*** | $3.50\pm6.0^a$ | $5.83\pm5.11^a$ | $6.17\pm5.35^a$ | $6.50\pm5.63^a$ | $48.50^b$ |
| ***Pseudomonas aeruginosa*** | 0.00 | $3.17\pm5.48^a$ | $7.33\pm6.43^a$ | $14.00\pm4.62^a$ | $41.50^b$ |
| ***Streptococcus mutans*** | $3.17\pm5.48^a$ | $3.50\pm6.06^a$ | $4.17\pm7.22^a$ | $14.00\pm3.97^a$ | $53.50^b$ |
| ***Staphylococcus aureus*** | $3.33\pm5.77^a$ | $4.00\pm6.93^a$ | $11.00\pm1.73^a$ | $34.17\pm42.72^a$ | $42.50^a$ |
| ***Salmonella typhi*** | $3.00\pm5.20^a$ | $3.33\pm5.77^a$ | $3.30\pm5.77^a$ | $22.33\pm19.44^a$ | $44.00^a$ |

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 |
| ***Escherichia coli*** | $2.67\pm4.62^a$ | $3.30\pm5.77^a$ | $7.33\pm6.35^a$ | $10.17\pm0.29^a$ | $48.50^b$ |
| ***Pseudomonas aeruginosa*** | 0.00 | $2.83\pm4.90^a$ | $6.17\pm5.35^a$ | $7.00\pm6.08^a$ | $41.50^b$ |
| ***Streptococcus mutans*** | 0.00 | $9.33\pm0.76^a$ | $11.30\pm3.6^a$ | $14.33\pm3.26^a$ | $53.50^b$ |
| ***Staphylococcus aureus*** | 0.00 | $3.00\pm5.20^a$ | $3.00\pm5.20^a$ | $16.67\pm12.85^a$ | $42.50^a$ |
| ***Salmonella typhi*** | $3.17\pm5.48^a$ | $3.17\pm5.48^a$ | $6.17\pm5.35^a$ | $32.17\pm1.26^a$ | $44.00^a$ |

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. There 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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# The Antibacterial Efficacy of *Annona muricata* (Linn) Leaves against Some Enteric Bacteria

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***Authors' contributions***

*This work was carried out in collaboration between both authors. Author EOD designed the study and wrote the protocol. Author KYA performed the statistical analysis and wrote the first draft of the manuscript. Author KYA managed the analyses of the study and the literature searches. Author EOD guided in the entire research. Both authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** This research was carried out to evaluate the susceptibility patterns of some enteric bacteria to aqueous, ethanol and methanol extracts of *Annona muricata* leaf.
**Study Design:** Experimental design.
**Place and Duration of Study:** Department of Microbiology, Federal University of Technology, Akure, Ondo State, Nigeria. Between January, 2019 and May, 2019.
**Methodology:** Extraction of bioactive components of leaves was done by maceration and phytochemical screening was carried out on the leaf extracts to determine the bioactive components present. The bacteria isolates were subjected to antibiotic sensitivity test using standard methods while the antibacterial activity of the plant extracts on human enteric bacteria was determined using agar well dilution method. *A. muricata* bark extracts were purified using column chromatography method. The minimum inhibitory and minimum bactericidal concentrations (MIC/MBC) of the extracts was performed using tube dilution technique.
**Results:** The phytochemical screening of leaf extracts showed presence of saponins, tannins, flavonoids, terpenoid and glycosides. Antibacterial activities of the leaf extracts revealed that

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aqueous, ethanol and methanol extracts had highest zone of inhibition of 22.00±0.58 mm, 24.67±0.33 mm and 24.00±0.58 mm respectively on *Salmonella typhi*. The minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) for ethanol extract ranged between 25-100 mg/ml and 50-200 mg/ml respectively while methanol extract ranged between 25-50mg/ml and 50-100 mg/ml respectively. Ciprofloxacin (CPX) was significantly (P≤0.5) the most active of all the antibiotics used against the test organisms (both clinical and typed isolates).
**Conclusion:** This research revealed that *A. muricata* leaf extracts possesses antibacterial activity against human enteric bacteria isolates used in this study. The commercial antibiotics were observed to be effective in inhibiting the test organisms. The purified extracts of *A. muricata* leaf showed higher inhibitory effect compared to crude extracts which can serve as ingredient for the production of novel antibacterial agents for the treatment of infections caused by enteric bacteria.

*Keywords: Annona muricata leaf; phytochemical constituents; antibacterial activity; food borne diseases; enteric bacteria.*

## 1. INTRODUCTION

Enteric bacteria are referred to as Gram-negative bacteria that are associated with gastrointestinal flora or diseases. These bacteria are rod-shaped and possess the following characteristics; they are facultative anaerobes, catalase positives, ferment glucose, oxidase-negative and possess the enterobacterial common antigen in the cell wall [1]. They are one of the major causes of food borne gastroenteritis in humans and remain an important health problem worldwide [2].

World Health Organization defined Foodborne disease as disease that are usually either infectious or toxic in nature caused by agents that enter the body through the ingestion of food [3]. Foodborne illness is usually caused by bacteria, parasites, viruses, toxins, metals, and prions [4]. WHO estimates that worldwide foodborne and waterborne diarrhoeal diseases all together kill about 2.2 million people annually [3].

Antibacterial resistant problems associated with commonly used antibiotics as well as the re-emergence of multi-antibiotic resistant strains of pathogens has become a possible threat to public health [5]. Medicinal plants are the major bio resource of drugs for both traditional and conventional system of medicine [6]. Natural products, especially those derived from plants, have been used to help mankind sustain its health since the dawn of medicine. Over the past century, the phytochemicals in plants have been a pivotal pipeline for pharmaceutical discovery. The importance of the active ingredients of plants in agriculture and medicine has stimulated significant scientific interest in the biological activities of these substances [7].

*A. muricata* whole plant is a deciduous tree and medicinal plant that belongs to the member of the *Annonaceae* (custard apple) family comprising approximately 130 genera and 2300 species [8]. *A. muricata* is widely known as soursop or prickly custard apple due to the sour and sweet taste of its fruit [9]. Different parts of *A. muricata* are widely used in traditional medicine of many countries to cure various ailments and diseases. The natives of Malaysia apply the leaf juice of *A. muricata* on the head to protect against fainting and they also use the leaves to treat cutaneous (external) and internal parasites. The juicy flesh-fruit of the soursop is a remedy for rheumatism, arthritic pain, fever, heart, and liver diseases, diarrhea, dysentery, malaria, parasites, skin rashes and worms as well as increasing breast milk after childbirth. The seeds are used as an anti-anthelmintic against external and internal worms and parasites. *A. muricata* is employed in tropical Africa as insecticidal and pesticidal agents besides being used for the treatment of coughs, pain and skin diseases [10]. Studies have shown that *A. muricata* contain saponins, condensed tannins and glycosides as the major constituents and trace amounts of flavonoids which contribute immensely to the bioactivity of the plant and also to its usage in treating various diseases [11]. Plants possess active phytoconstituents that can confer antimicrobial activities against pathogenic organism with minimal or no side effect. Some pathogens use the host immune response for spreading the infection. Thus, requires the urgency in finding new methods of controlling enteric diseases and hence exploring plants as alternatives. Therefore, it is of great interest to assess the susceptibility patterns of some enteric bacteria to leaf extracts of *Annona muricata.*

## 2. METHODOLOGY

### 2.1 Plant Collection and Identification

Fresh leaves of *A. muricata* L. were collected during the raining season from a garden at Adebayo, Ado Ekiti, Ekiti State. The leaves were identified and authenticated at the Department of Crop, Soil and Pest management, Federal University of Technology, Akure (FUTA).

### 2.2 Extraction of *A. muricata* Bark

The leaves of *A. muricata* were washed with sterile water, air dried for three weeks and pulverized into fine powder. Fifty grams (50 g) of the powdered leaves was soaked into one liter (1L) of cold water, 100% ethanol and methanol (BDH England). The container of the mixtures were labeled and left covered for 3 days (72 hours) with intermittent agitation followed by sieving with a muslin cloth and filtered using No 1 Whatman filter paper (UK). The filtrates were vaporized to dryness using rotary evaporator (RE-52A Union laboratories England) and subsequently lyophilized to remove the extracting solvent. The crude extracts obtained was preserved in a sterile container and stored in the refrigerator at 4°C until when ready for use [5]. The crude extracts which were obtained were sterilized using 0.45µm millipore membrane filter in order to remove any contaminant that might be present before subsequent use.

The weight of the dried extracts was measured and the percentage recovery was calculated as;

Percentage recovery = (Weight of extract recovered after extraction×100%)/Initial weight of plant part

### 2.3 Purification of *A. muricata* Leaf Extracts

The leaf extracts were purified according to the method described by [12]. The column was packed with silica gel of 60-120 mesh. During this process of packing the gel, utmost care was taken in other to avoid distortion and cracking of the gel. A 250 ml burette was attached to a retort stand; small piece of glass wool was tucked down lightly to avoid particles from the cotton dropping into the fraction during separation into the burette with the aid of an applicator stick. For the mobile phase 1: 1: 1 proportions of three solvents were used. 100 g of silica gel was mixed thoroughly with equal volume of Methanol, Ethanol and Acetone and poured into the burette. A 100 ml of the solvents was used to top the silica gel for it to flow down slowly to allow the proper packing of the column. Two (2 g) grams of the crude extracts was mixed with 5 ml of the solvents and added carefully unto the surface of the column. More solvent was added as the fractions of the extracts were being obtained in small sterile containers.

### 2.4 Phytochemical Screening of *A. muricata* Leaf Extracts

The aqueous, ethanol and methanol leaf extracts of *A. muricata* were subjected to qualitative and quantitative phytochemical screening for the presence of bioactive constituents such as tannins, phenols, alkaloids, glycosides, anthroquinones, saponins and flavonoids using the method described by AOAC [13].

#### 2.4.1 Test for alkaloids

Five millimeters (5 ml) of 1% aqueous hydrochloric acid (HCl) was added to 0.5 g of the extracts in test tubes and put in a water bath for 2 minutes, after which the mixture was filtered. 1 ml of the filtrate was treated with three drops of Dragendroffs reagent. The presence of alkaloids was confirmed by the production of blue black turbid colouration.

#### 2.4.2 Test for tannins

About 0.5 g of the plant extracts were stirred in distilled water and filtered. Ferric chloride (0.1% $FeCl_3$) reagent was added to the filtrate. A blue black or blue green precipitate was taken as preliminary evidence for presence of tannin.

#### 2.4.3 Test for steroids

Two milliliters (2 ml) of acetic anhydride was added to 0.5 g of the extract of each sample with addition of 2 ml of sulphuric acid ($H_2SO_4$). A colour change from violet to blue or green indicates the presence of steroids.

#### 2.4.4 Test for saponins

The ability of Saponins to produce frothing in aqueous solution was used as screening test for saponins. 0.5 g of the extract was boiled with distilled water in a water bath and shaken vigorously for a stable persistent froth. The frothing was mixed with three drops of olive oil and shaken vigorously, then observed for the formation of emulsion.

### 2.4.5 Test for phlobatannins

Extracts of *A. muricata* was boiled with 1% HCI (aqueous). The formation of red precipitate was taken as preliminary evidence of phlobatannin.

### 2.4.6 Test for anthraquinone

About 0.5 g of the extract was shaken with 10ml of benzene, filtered and then 5 ml of 10% ammonia solution was added to the filtrate and the mixture was shaken. The presence of red, pink or violet colour in the ammonia (lower) layer indicates the presence of free anthraquinone.

### 2.4.7 Test for flavonoids

Ten milliliter (10 ml) of ethyl acetate was heated with plant extracts in a water bath for thirty minutes. The mixture was filtered and four milliliters (4 ml) of each filtrate was shaken with one milliliter (1 ml) of dilute ammonia solution in a conical flask. A yellow colouration indicates the presence of flavonoids.

### 2.4.8 Test for cardiac glycosides

The following tests were carried out to determine cardiac glycosides.

#### *2.4.8.1 Legal's test*

The extract was dissolve in pyridine and a few drops of 2% sodium nitroprusside together with few drops of 20% sodium hydroxide (NaOH) were added. A deep red colouration which faded to a brownish yellow indicates the presence of cardenolides.

#### *2.4.8.2 Lieberman's test*

Two milliliter (2 ml) of acetic anhydride was added to 0.5 g of the extract and filter, 2 ml of concentrated sulphuric acid ($H_2SO_4$) was to the filtrate. There was a colour change from violet to blue, green which indicate the presence of steroids nucleus i.e. aglycone portion of the cardiac glycoside.

#### *2.4.8.3 Salkowski's test*

Two milliliter (2 ml) of chloroform was used to dissolve 0.2 g of the extracts. Concentrated sulphuric acid was carefully added which formed a lower layer. A reddish-brown colour at the interface was observed which indicate the presence of steroidal ring.

#### *2.4.8.4 Keller-killiani's test*

Two milliliter (2 ml) of glacial acetic acid containing one drop of ferric chloride ($FeCl_3$) solution was used to dissolve 0.2 g of the plant extracts. The mixture was under layered with 1ml concentrated sulphuric acid ($H_2SO_4$). A brown ring obtained at the interface indicates the presence of a deoxy-sugar. Quantitative phytochemical screening was carried out using spectrophotometer (Model 721G) [13].

## 2.5 Collection of Bacterial Isolates

Clinical enteric bacteria isolates (*Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris Pseudomonas aeruginosa, Salmonella typhi* and *Shigella dysenteriae*) were obtained from the stock culture of Ekiti State University Teaching Hospital, Ado Ekiti, Ekiti State and the typed enteric bacteria isolates (Escherichia coli ATCC 25922, *Klebsiella pneumonia* ATCC 13883, *Proteus vulgaris* ATCC 29905, *Pseudomonas aeruginosa* ATCC 10145, *Salmonella typhi* ATCC 14028) was obtained from Federal Institute of Research, Oshodi (FIRO). The bacteria isolates were kept on already prepared nutrient agar slants and transported immediately to the microbiology laboratory of the Federal University of Technology, Akure, Ondo State for further analysis. These organisms were confirmed by biochemical tests.

## 2.6 Determination of the Antibacterial Activity of *A. muricata* Leaf Extracts

The sensitivity of each of the extracts was determined using agar well diffusion. The ethanol, aqueous and methanol extracts was reconstituted with 30% Dimethyl sulfoxide (DMSO) to obtain varying concentration [14].

The bacterial isolates were grown in nutrient broth and adjusted to 0.5 McFarland's standard solution. The standardized bacteria isolates were aseptically inoculated on the surface of already prepared sterile Mueller-Hilton agar plate by means of sterile cotton swab making sure they were evenly spread on the surface of the agar plate. This procedure was repeated by streaking two times, rotating the plate approximately 60° each time to ensure an even distribution of the inoculums. The agar wells were bored using a sterile corkborer with 6 mm diameter on the solidified agar medium. 200 mg/ml of the leaf extract was prepared using a reconstituting solvent of 30% Dimethyl sulfoxide (DMSO). 0.2

ml of each of the extracts was carefully added into the wells of labeled plates and holes. The plates were allowed to stand on the work bench for 1 hr to allow proper inflow of the extract into the medium before incubation. Plates were incubated in an upright position at 37°C for 24 hrs. DMSO was used as the negative control while ciprofloxacin was used as positive control. After overnight incubation, zones of inhibition formed on the surface of the plates were measured in millimeter [15].

### 2.7 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Leaf Extract

The MIC and MBC of the extracts was carried out using tube dilution technique. To 1 ml of graded concentrations (200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml) of the extracts, 10 ml of 24 hrs Mueller-Hinton broth was added and a loopful of test organisms previously diluted was introduced into the tubes. Ciprofloxacin was included as positive control and distilled water as negative control in different tubes. A tube containing only nutrient broth was seeded with test organism to serve as positive control while a tube that was not inoculated served as the negative control. All the broth cultures were incubated at 37°C for 24 hrs. After incubation the tubes were examined for microbial growth by observing for turbidity using spectrophotometer. Growth inhibition was indicated by low turbidity while growth was indicated by high turbidity. From each of the set of test tubes used for the determination of MIC, a loopful of broth was collected from the tubes that do not show any visible growth and was inoculated on sterile Mueller-Hinton agar plates. The plates were incubated at 37°C for 24 hrs. After incubation, the least concentration that showed no growth was recorded as the minimum bactericidal concentration (MBC) [15].

### 2.8 Antibiotic Sensitivity Pattern

Antibiotic sensitivity testing was performed using disc diffusion method. Standard antibiotic discs for gram negative bacteria were used against the bacteria isolates. These antibiotics include pefloxacin 30 µg (PEF), gentamycin 30 µg (CN), augmetin 10 µg (AU), sparfloxacin 10 µg (SP), amoxacillin 30 µg (AM), chloramphenicol 30 µg (CH), ciprofloxacin 30 µg (CPX), streptomycin 30 µg (S), septrin 30 µg (SXT) and tarivid 10 µg (OFX). The inoculum was prepared by emulsifying three to four discrete colonies of each test isolate in a sterile test tube containing peptone water and incubated for 30 minutes. The suspension was adjusted to match with 0.5 McFarland turbidity standards after which the peptone water isolate suspension was poured into a freshly prepared Mueller-Hilton agar plate and swirled gently to cover the surface of the agar. Then, the antibiotic discs was placed aseptically on the surface of the inoculated plate using a sterile forceps and pressed lightly to ensure contact with the agar surface. The plate was incubated at 37°C for 24hours. After incubation, clear zones of inhibition were measured in millimeter and areas without clear zones were observed. Inoculated plate without antibiotics served as control [16].

### 2.9 Statistical Analysis

Data obtained were subjected to One Way Analysis of Variance (ANOVA) while the means were compared with Duncan's New Multiple Range Test at 95% confidence interval using Statistical Package for Social Sciences version 23.0. Differences were considered significant at $p \leq 0.05$.

## 3. RESULTS

### 3.1 Percentage Recovery of Aqueous, Ethanol and Methanol Leaf Extracts of *A. muricata*

Table 1 revealed the percentage recovery of the leaf extracts of *A. muricata* after extraction. The table showed that more extracts was recovered with methanol and ethanol than water with 55.36%, 50.80% and 41.08% respectively. Methanol extract has the highest percentage yield of 55.36%.

**Table 1. Percentage recovery of leaf extracts of *A. muricata***

| Solvents | Percentage (%) |
|---|---|
| Aqueous | 41.08% |
| Ethanol | 50.80% |
| Methanol | 55.36% |

### 3.2 Qualitative and Quantitative Phytochemical Screening of *A. muricata* Leaf Extracts

Qualitative phytochemical screening of the aqueous, ethanol and methanolic extracts of *A.*

*muricata* leaf is presented in Table 2. The results revealed that saponin, tannin, flavonoid, terpenoid and cardiac glycosides were present in the aqueous, ethanol and methanol leaf extracts of *A. muricata* while phlobatannin, alkaloid and anthraquinone were absent in all the plant extracts. However, the presence of Steroid was recorded only for methanol extract but absent in other crude extracts of the plant.

Table 3 showed the quantitative phytochemical (mg/100g) composition of aqueous, ethanol and methanol crude extracts obtained from leaf of *A. muricata*. The result revealed that there was significant difference (P<0.05) in the quantity of saponin, tannin, terpernoid, glycosides, flavonoid and steroid composition of crude extracts obtained from the leaves but there was no significant difference in the alkanoid and phlobatannin composition of the crude extracts. For aqueous extract, saponin had the highest quantity (56.09±0.27 mg/g) while tannin had the least quantity (5.09±3.34 mg/g). Glycosides had the highest quantity (19.59±0.05 mg/g) in ethanol extract while Tannin had the least quantity (7.17±0.01 mg/g) in methanol extract.

### *3.3* Antibacterial Activity of *A. muricata* Leaf Extracts

Table 4 shows the susceptibility patterns of the bacteria isolates to aqueous, ethanol and methanol leaf extracts (crude) of *A. muricata* at 200 mg/ml.

Aqueous leaf extract does not show zone of inhibition on all the test organisms (both clinical and typed) except for *S. typhi*, *E. coli* and *K. pneumoniae* with the highest zone of inhibition on *S. typhi* (22.00 mm) with least inhibitory activity on *E. coli* (12.33 mm). Ethanol leaf extract showed inhibition diameter ranging from (14.33 mm to 24.67 mm) with the highest inhibitory activity on *S. typhi* (24.67 mm) and least inhibitory activity on *P. aeruginosa and E. coli* (14.33mm) respectively. Ethanol leaf extract did not inhibit *Shigella sp*. All the bacteria isolates (both clinical and typed) were susceptible to methanol extract with zones of inhibition ranging from (14.00 mm to 24.00 mm). Methanol leaf extract was more effective on the test organisms than cold water and ethanol extracts.

**Table 2. Qualitative phytochemical screening of aqueous, ethanol and methanol leaf extract of *A. muricata***

| | Aqueous | Ethanol | Methanol |
|---|---|---|---|
| Saponin | + | + | + |
| Tannin | + | + | + |
| Phlobatannin | - | - | - |
| Flavonoid | + | + | + |
| Steroid | - | - | + |
| Terpenoid | + | + | + |
| Alkaloid | - | - | - |
| Anthraquinone | - | - | - |
| **Cardiac glycosides** | | | |
| Legal test | + | + | + |
| Keller kiliani test | + | + | + |
| Salkwoski test | + | + | + |
| Lieberman test | - | - | + |

**Table 3. Quantitative phytochemical composition of *Annona muricata* leaf crude extract**

| Phytochemical | Aqueous (mg/g) | Ethanol (mg/g) | Methanol (mg/g) |
|---|---|---|---|
| Saponin | $56.09\pm0.27^{c}$ | $16.82\pm0.27^{a}$ | $26.82\pm0.27^{b}$ |
| Tannin | $5.09\pm3.34^{b}$ | $3.34\pm0.01^{a}$ | $7.17\pm0.01^{c}$ |
| Terpenoid | $16.42\pm0.04^{c}$ | $13.36\pm0.04^{b}$ | $10.12\pm0.04^{a}$ |
| Glycosides | $10.59\pm0.05^{a}$ | $19.59\pm0.05^{c}$ | $16.96\pm0.05^{b}$ |
| Flavonoid | $11.34\pm0.02^{b}$ | $6.95\pm0.02^{a}$ | $13.04\pm0.03^{c}$ |
| Alkanoid | $0.00\pm0.00^{a}$ | $0.00\pm0.00^{a}$ | $0.00\pm0.00^{a}$ |
| Steroid | $0.00\pm0.00^{a}$ | $6.74\pm0.02^{b}$ | $0.00\pm0.00^{a}$ |
| Phlobatannin | $0.00\pm0.00^{a}$ | $0.00\pm0.0^{a}$ | $0.00\pm0.00^{a}$ |

*Data are represented as mean ± standard error (n=3). Superscript of the same alphabet in the same row are not significantly different (P<0.05) while different alphabet denotes significant difference*

**Table 4. Antibacterial activity of leaf extract (Crude) at 200 mg/ml**

| Isolates | Aqueous | Ethanol | Methanol | Control |
|---|---|---|---|---|
| *Pseudomonas aeruginosa* | 0.00±0.00$^{a}$ | 14.33±0.33$^{b}$ | 15.33±0.33$^{c}$ | 34.33±0.33$^{d}$ |
| *Salmonella typhi* | 22.00±0.58$^{a}$ | 24.67±0.33$^{b}$ | 24.00±0.58$^{b}$ | 39.00±0.58$^{c}$ |
| *Shigella sp* | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 14.67±0.33$^{b}$ | 34.00±0.58$^{c}$ |
| *Escherichia coli* | 12.33±0.33$^{a}$ | 14.33±0.33$^{b}$ | 17.33±0.33$^{c}$ | 21.00±0.58$^{a}$ |
| *Klebsiella pneumoniae* | 20.33±0.33$^{a}$ | 22.33±0.67$^{b}$ | 24.00±0.58$^{b}$ | 35.00±0.58$^{c}$ |
| *Proteus vulgaris* | 0.00±0.00$^{a}$ | 15.00±0.58$^{b}$ | 14.00±0.58$^{b}$ | 31.00±0.58$^{c}$ |
| Typed *Proteus vulgaris* | 0.00±0.00$^{a}$ | 17.33±0.58$^{b}$ | 21.67±0.33$^{c}$ | 38.33±0.88$^{d}$ |
| Typed *Pseudomonas aeruginosa* | 0.00±0.00$^{a}$ | 19.33±0.33$^{b}$ | 22.00±0.58$^{c}$ | 35.00±0.58$^{d}$ |
| Typed *Salmonella typhi* | 0.00±0.00$^{a}$ | 17.33±0.33$^{b}$ | 18.33±0.67$^{b}$ | 29.00±0.58$^{c}$ |
| Typed *Escherichia coli* | 0.00±0.00$^{a}$ | 17.00±0.58$^{b}$ | 17.33±0.33$^{b}$ | 28.33±0.33 $^{c}$ |
| Typed *Klebsiella Pneumoniae* | 0.00±0.00$^{a}$ | 18.33±0.58$^{b}$ | 19.67±0.33$^{c}$ | 26.33±0.33$^{d}$ |

*Data are represented as mean ± standard error (n=3). Superscript of the same alphabet in the same row are not significantly different (P<0.05) while different alphabet denotes significant difference*

**Table 5. Antibacterial activity of leaf extract (Purified) at 200 mg/ml**

| Isolates | Ethanol | Methanol | Control |
|---|---|---|---|
| *Pseudomonas aeruginosa* | 16.33±0.33$^{a}$ | 18.33±0.67$^{a}$ | 35.00±1.00$^{b}$ |
| *Salmonella typhi* | 26.00±0.58$^{a}$ | 25.67±0.33$^{a}$ | 38.33±0.33$^{b}$ |
| *Shigella sp* | 9.33±0.33$^{a}$ | 16.33±0.33$^{b}$ | 34.33±0.88$^{c}$ |
| *Escherichia coli* | 15.33±0.67$^{a}$ | 18.33±0.33$^{b}$ | 21.33±0.88$^{c}$ |
| *klebsiella pneumoniae* | 24.00±0.58$^{a}$ | 26.00±0.58$^{a}$ | 35.33±0.88$^{b}$ |
| *Proteus vulgaris* | 18.00±0.58$^{a}$ | 16.67±0.88$^{a}$ | 32.00±0.58$^{b}$ |
| Typed *Proteus vulgaris* | 19.67±0.33$^{a}$ | 23.33±0.33$^{b}$ | 38.00±0.58$^{c}$ |
| Typed *Pseudomonas aeruginosa* | 21.00±0.58$^{a}$ | 24.00±0.58$^{b}$ | 34.67±0.33$^{c}$ |
| Typed *Salmonella typhi* | 18.00±0.58$^{a}$ | 19.33±0.67$^{a}$ | 28.67±0.88$^{b}$ |
| Typed *Escherichia coli* | 17.67±0.67$^{a}$ | 19.33±0.33$^{a}$ | 27.00±0.58$^{b}$ |
| Typed *Klebsiella Pneumoniae* | 20.33±0.88$^{a}$ | 20.67±0.88$^{a}$ | 27.33±0.33$^{b}$ |

*Data are represented as mean ± standard error (n=3). Superscript of the same alphabet in the same row are not significantly different (P<0.05) while different alphabet denotes significant difference*

Table 5 revealed the result of susceptibility patterns of the bacteria isolates to ethanol and methanol leaf extracts (purified) of *A. muricata* at 200mg/ml. The purified leaf extracts showed increase in the inhibitory effect of the plant against all isolates. There was a significant increase in the ethanol and methanol activities of the leaf extracts on the bacteria isolates compared to the crude extracts.

The purified ethanol extract showed highest inhibitory effect against *S. typhi* with zone of 26.00 mm while purified methanol extract inhibited the bacteria isolates with a varied range from (18.33 mm to 26.00 mm) with the highest zone on *K. pneumoniae* and least zone on *E. coli* (18.33 mm).

### 3.4 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Leaf Extracts

Table 6 shows the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of *A. muricata* leaf extracts on test organisms (clinical and typed bacteria isolates).

The MIC of ethanol extract ranged from 25 to 100 mg/ml while that of methanol extract ranged from 25 to 50 mg/ml. The MIC for ethanol extract of the plant showed that *P. aeruginosa* and *K. pneumoniae* had the highest MIC at 100 mg/ml while methanol extract of the plant showed the lowest MIC of 25 mg/ml on *E. coli, Shigella* sp*, S. typhi*, *P. vulgaris* ATCC 29905 and *K. pneumoniae* ATCC 13883.

Also, the (MBC) of the ethanol and methanol leaf extract of *A. muricata* ranged from 50-200mg/ml. The MBC for ethanol extract of the plant showed that *P. vulgaris, P. aeruginosa* ATCC 10145, *K. pneumoniae* ATCC 13883 and *S. typhi* ATCC 14028 had the lowest MBC at 50 mg/ml while methanol extract showed the highest MBC at 200 mg/ml on *K. pneumoniae.*

### 3.5 Antibiotic Sensitivity Pattern

Table 7 shows the antibiotics sensitivity patterns of the Gram negative bacterial isolates to conventional antibiotics. All the isolates were susceptible to ciprofloxacin (CPX) and Pefloxacin (PEF) with the highest diameter zone of inhibition (29.00mm) on *P*. *aeruginosa* ATCC 10145 and *Shigella* Sp (25.67 mm) respectively. *S*. *typhi* showed total resistance to Gentamycin (CN) while others were susceptible with highest diameter zone of inhibition (19.67 mm) on *E*. *coli.* Only *K*. *pneumoniae* was resistant to Tarivid (OFX) while others were susceptible with the highest diameter zone of inhibition (23.67 mm) on *Shigella* sp.

## 4. DISCUSSION

*Annona muricata* are extensively used to combat worms and parasitic organisms, to cool fevers, and as an astringent for diarrhea and dysentery [5]. Findings from this study revealed that methanol had the highest extraction yield (55.36%). This agrees with the findings of [17] who reported highest yield in methanol bark extract of *Annona muricata.*

Phytochemical screening of the crude extracts of *A. muricata* leaf revealed the presence of some bioactive components such as saponin, tannin, flavonoid, terpenoid and cardiac glycosides. This is in agreement with the work of Muhammad *et al*., [18] who reported similar bioactive compounds in the leaves of *Carica papaya*. The presence of saponin, tannin, flavonoid, terpenoid and cardiac glycosides may be attributed to different solvents used in the extraction of the bioactive components [19] and probably account for the antibacterial activity of the extracts.

The susceptibility patterns of clinical and typed enteric bacteria isolates to leaf extracts (crude) of *A. muricata* at 200 mg/ml showed variations in the zone of inhibition for each extracts. Methanol leaf extract was found to be more effective in inhibiting the test organisms than aqueous and ethanol extract. This result is in line with Adamu et al. [20] who reported a significant increase in microbial activities of methanol extract of *A. muricata*. Clinical isolates were observed to be more susceptible to both ethanol and methanol extracts of the plant than the typed isolates. This may be because the clinical isolates have not been previously exposed to antibacterial agents that could have generated resistance to the extracts and antibiotics used in this study [17]. Ethanol and methanol crude extracts of *A. muricata* leaf showed antibacterial activity against several of the test organisms used in this study. This may be due to the fact that water contains a lot of organic and inorganic compounds which may or may not interact to inhibit their overall activities [16]. The responses of the organisms to the crude extracts as revealed by the observed zone of inhibition might be due to the presence of metabolites revealed through the phytochemical screening, which possess pharmacological activities responsible for the use of plants in traditional phytomedicine to treat diseases caused by enteric bacterial [21].

The purified extracts of *A. muricata* leaf showed that there was increase in the inhibitory activities of ethanol and methanol leaf extracts on the test organisms. This result is in line with the study of Dada and Akinde [17] who attested that the purified leaf extracts *A. muricata* showed higher antibacterial effect on tested bacterial isolates compared to the crude extracts. This result suggests that they have remarkable therapeutic action in the treatment of enteric diseases.

**Table 6. Minimum inhibitory MIC (mg/ml) and minimum bactericidal concentration MBC (mg/ml) for leaf extract (mg/ml)**

| Organisms | MIC ethanol | Methanol | MBC ethanol | Methanol |
|---|---|---|---|---|
| *Proteus vulgaris* | 25 | 50 | 50 | 100 |
| *Klebsiella pneumoniae* | 100 | 50 | 200 | 200 |
| *Pseudomonas aeruginosa* | 100 | 50 | 200 | 100 |
| *Escherichia coli* | 50 | 25 | 100 | 50 |
| *Shigella sp* | 50 | 25 | 200 | 50 |
| *Salmonella typhi* | 25 | 25 | 100 | 50 |
| Typed *Proteus vulgaris* | 50 | 25 | 100 | 50 |
| Typed *Pseudomonas aeruginosa* | 25 | 50 | 50 | 100 |
| Typed *Klebsiella pneumoniae* | 25 | 25 | 50 | 100 |
| Typed *Escherichia coli* | 50 | 50 | 100 | 100 |
| Typed *Salmonella typhi* | 25 | 50 | 50 | 100 |

**Table 7. Antibiotics sensitivity patterns showing diameter of zone of inhibition**

| Bacteria | PEF | OFX | S | SXT | CH | SP | CPX | AM | AU | CN |
|---|---|---|---|---|---|---|---|---|---|---|
| EcC | 25.33±0.58$^{g}$ | 21.33±0.67$^{e}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 25.67±0.67$^{h}$ | 24.00±0.58$^{fg}$ | 17.50±0.58$^{e}$ | 14.33±0.33$^{c}$ | 19.67±0.33$^{e}$ |
| KpC | 20.33±0.58$^{de}$ | 0.00±0.00$^{a}$ | 14.67±0.58$^{c}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 15.67±0.67$^{c}$ | 14.00±0.58$^{b}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 17.33±0.33$^{d}$ |
| PaC | 16.67±0.58$^{c}$ | 20.33±0.58$^{e}$ | 20.00±0.58$^{e}$ | 21.67±0.33$^{f}$ | 20.00±0.58$^{d}$ | 21.67±0.88$^{f}$ | 23.00±0.58$^{f}$ | 15.00±0.58$^{d}$ | 19.67±0.67$^{d}$ | 18.00±0.58$^{d}$ |
| StC | 20.33±0.58$^{de}$ | 20.00±0.58$^{d}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 24.67±0.33$^{fg}$ | 0.00±0.00$^{a}$ | 0.00±0.00 | 0.00±0.00$^{a}$ |
| SsC | 25.67±0.58$^{g}$ | 23.67±0.57$^{f}$ | 22.67±0.33$^{g}$ | 14.67±0.33$^{c}$ | 23.00±0.58$^{e}$ | 17.00±0.58$^{de}$ | 27.00±0.33$^{h}$ | 16.00±0.58$^{de}$ | 19.33±0.33$^{d}$ | 19.33±0.33 |
| PvC | 14.67±0.58$^{b}$ | 14.33±0.58$^{b}$ | 15.00±0.58$^{c}$ | 20.33±0.33$^{e}$ | 0.00±0.00$^{a}$ | 14.33±0.33$^{b}$ | 18.33±0.89$^{c}$ | 12.00±0.58$^{b}$ | 0.00±0.00$^{a}$ | 10.33±0.33$^{b}$ |
| KpT | 19.00±0.58$^{de}$ | 19.33±0.57$^{d}$ | 14.67±0.33$^{c}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 14.33±0.33$^{b}$ | 19.00±0.89$^{d}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 16.67±0.33$^{d}$ |
| PvT | 21.00±0.58$^{d}$ | 16.33±0.58$^{c}$ | 17.00±0.58$^{e}$ | 18.33±0.67$^{d}$ | 0.00±0.00$^{a}$ | 18.33±0.33$^{e}$ | 21.33±0.67$^{e}$ | 17.00±0.58$^{e}$ | 0.00±0.00$^{a}$ | 14.00±0.33$^{c}$ |
| PaT | 23.33±0.67$^{f}$ | 15.00±0.58$^{b}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 15.00±0.58$^{c}$ | 13.67±0.33$^{b}$ | 29.00±0.58$^{i}$ | 11.00±0.58$^{b}$ | 12.00±0.58$^{b}$ | 14.33±0.58$^{c}$ |
| EcT | 23.33±0.58$^{f}$ | 20.33±0.58$^{de}$ | 16.00±0.58$^{e}$ | 14.67±0.33$^{c}$ | 15.00±0.58$^{c}$ | 22.33±0.33$^{g}$ | 27.00±0.58$^{h}$ | 17.00±0.58$^{e}$ | 13.67±0.33 | 17.33±0.88$^{d}$ |
| StT | 15.00±0.58$^{b}$ | 15.00±0.58$^{b}$ | 13.33±0.33$^{b}$ | 11.67±0.33$^{b}$ | 13.67±0.33 | 13.67±0.33$^{b}$ | 17.00±0.58$^{c}$ | 13.33±0.33$^{c}$ | 0.00±0.00$^{a}$ | 13.67±0.33$^{c}$ |

*Data are represented as mean ± standard error (n=3). Superscript of the same alphabet in the same row are not significantly different (P<0.05) while different alphabet denotes significant difference. Legend: PEF= Pefloxacin, OFX: Tarivid, S: Streptomycin, SXT= Septrin, CH= Chloramphenicol, SP= Sparfloxacin, CPX= Ciprofloxacin, AM= Amoxicillin, AU= Augmetin, CN= Gentamycin. EcC= E. coli, KpC= K. pneumoniae, PaC= P. aeruginosa, StC= S. typhi, SsC= Shigella. sp, PvC= P. vulgaris, KpT= K. pneumoniae ATCC 13883, PvT= P. vulgaris ATCC 29905, PaT= P. aeruginosa ATCC 10145, EcT= E. coli ATCC 25922, StT= S. typhi ATCC 14028*

The minimum inhibitory concentration (MIC) of leaf extracts against clinical and typed isolates was found to be (25 mg/ml) while the MBC was found to be (50 mg/ml). The result of this study revealed that the minimum bactericidal concentration (MBC) of the plant extracts indicated higher concentrations than that of the MIC. This observation is based on the fact that the concentration of the extracts required to completely eliminate an organism must be higher than the concentration required to inhibit the growth [22].

The commercial antibiotics were observed to be effective in inhibiting the test organisms (both clinical and typed isolates). Ciprofloxacin (CPX) was the most effective against the test organisms (both clinical and typed isolates) out of all the antibiotics used. The high inhibition values recorded by antibiotics than the plant extracts on clinical and typed isolate may be due to its purified nature, as reported by [15] that antibiotics are in a refined state while plant extracts are still in crude state. Also, the small molecular size possessed by antibiotics as reported by Mailard [23] aids their solubility in diluents as this could enhance their penetration through the cell wall into the cytoplasm of the organism.

When susceptibility patterns of the bacteria isolates were compared to standard antibiotics and extracts of *A. muricata*, it was deduced that for both clinical and typed isolates, the activity of the extracts will compete favourably with the standard antibiotics in the treatment of enteric bacterial infection. However, the differences observed between the activities of the standard antibiotics and extracts of *A. muricata* might be due to the proportion of the bioactive components present in the extracts.

## 5. CONCLUSION

This study has revealed *A. muricata* leaf extracts to be rich in flavonoids, tannins, saponins, terpenoid and cardiac glycosides as secondary metabolites which is responsible for the various antibacterial activities exhibited. The result showed that ethanol and methanol extracts exhibited considerable inhibitory activity against the test organisms used as demonstrated by the diameter zones of inhibition. Among the different solvents used, methanol extract showed the highest antibacterial activity**.** Ciprofloxacin had inhibitory effect on the tested bacterial isolates. The purified leaf extracts of *A. muricata* had higher antibacterial activity on the test organisms. However, the purified ethanol leaf extracts can serve as ingredient for the production of novel antibacterial agents which can be used for the treatment of infections caused by enteric bacteria. Thus, the need for identification of the active components contained in the leaf extracts and also ascertain the biosafety of the plant part.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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# Phytochemical screening and *in vitro* antimicrobial properties of *Annona muricata* extracts against certain human pathogens

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**Key words:** *Annona muricata*, pathogens, bacteria, fungi, antibiotics, medicinal plants

**Abstract**

All over the world, the use of medicinal plants is gaining more acceptability due to the possibility of discovering novel drugs from them and solving the problem of antimicrobial resistance associated with conventional antibiotics. The phytochemical composition and antimicrobial properties of crude extracts of the leaves, stems, and bark of *Annona muricata* were evaluated on *Escherichia coli, Staphylococcus aureus,* and *Salmonella typimurium*, while the antifungal properties were evaluated against *Candida albicans* and *Candida tropicalis*. The Agar well method was used for the study. At concentrations of 150 mg/ml and 300 mg/ml, inhibitory effects were observed on *E. coli* and *S. aureus*, with a visible zone of inhibition ranging from 15 mm to 21 mm respectively, and with respect to N- hexane, an antimicrobial activity range of 5 mm to 20 mm, for the leaf extract, which shows effective antimicrobial action against *E. coli* and *S. aureus*. Hot water extracts were observed to possess more bioactive compounds compared to organic solvent extracts, and exhibit higher ranges of activity against the tested bacterial species. All extracts exhibited low anti-fungal activity in the range of 8 mm to 15 mm. The phytochemical screening of the extracts of different parts of *A. muricata* revealed the presence of secondary metabolites such as tannins, alkaloids, saponins, flavonoids, steroids, and cardiac glycosides. The antimicrobial activity of the extracts was compared with a standard antibiotic, ketoconazole, and with ampicillin, which served as the controls). The results showed that *A. muricata* can be used as an anti-bacterial substance, since it shows broad spectrum activity against a range of bacteria responsible for the most common bacterial illnesses. Further research will be necessary to ascertain its full spectrum of efficacy.

## Introduction

All over the world, herbal medicine has served as perhaps the most valuable and popular field of traditional medicine. Medicinal plants have been used to treat illnesses since before recorded history (Gajalakshmi, Vijayalakshmi & Rajeswari, 2012). The study of medicinal plants is essential to promoting proper use of herbal medicines and in order to identify potential sources of new drugs (Parekh & Chanda, 2007). According to a World Health Organization (WHO) report, greater than 80% of the world's population depends on traditional medicine to satisfy their primary health care needs (Vashist & Jindal, 2012). Finding new naturally active components of plants and plant-based products has interested many scientific researchers. In this regard, the antimicrobial properties of botanicals have attracted a great deal of attention as a promising potential source of novel pharmaceutical drugs.

Soursop is one of the medicinal plants reported to have properties beneficial to health. Its scientific name is *Annona muricata* (Sarah, Mustafa & Rehab, 2015). *Annona muricata*, commonly known as graviola or soursop, belongs to the family of *Annonaceae*. It is a typical tropical tree, with heart-shaped edible fruits and widely distributed in most tropical countries (Foong & Hamid, 2012). It is a small tree, native to and widespread in Central America and the Caribbean, but now also widely

cultivated, and in some areas becoming invasive, in tropical locales throughout the world (Le Ven et al., 2011; Moghadamtousi et al., 2015). It has become an important crop because of its tasty flavor, high pulp content, nutritional value, and antioxidant properties (Moghadamtousi et al., 2015). The plant has various native names, depending on the country where it is found. It is called Guanabana in China and, in Nigeria, it is proudly called Shawahopu in the Igbo language (Le Ven et al., 2011; Moghadamtousi et al., 2015).

Gajalakshimi et al. (2012) reports that *A. muricata* is a traditional medicinal plant with phytochemical constituents and bioactive compounds possessing diverse medicinal properties. Intensive chemical investigation of the leaves and seeds of the species have resulted in the isolation of a great number of acetogenins (Moghadamtousi et al., 2015). The isolated compounds display some desired biological and pharmacological effects such as anti-tumoral properties, cytoxicity, and pesticidal properties (Moghadamtousi et al., 2015). These conclusions are supported by the use in traditional medicine of the roots of the species for their anti-parasitical and anti-pesticidal properties (Moghadamtousi et al., 2015; Sarah, Mustafa & Rehab, 2015). *A. muricata* has been traditionally used to treat headaches, hypertension, cough, and asthma and used as an antispasmodic, sedative, and nervine for heart conditions (Sarah, Mustafa & Rehab, 2015).

Soursop leaves contain flavonoids, tannins, alkaloids, saponins, calcium, phosphorus, carbohydrates, vitamins A, B, and C, phytosterol, and calcium oxalate (Edeoga, Okwu & Mbaebie, 2005; Abdul Wahab et al., 2018). The leaves are traditionally used to prevent and treat asthma, bronchitis, biliary disorder, diabetes, heart diseases, hypertension, worm disease, liver disorder, malaria, rheumatism, arthritis, other sources of joint pain, tumors, and cancer (Padma et al., 2001; Wicaksono et al., 2011). The leaves are also used to treat several types of bacterial disease, such as pneumonia, diarrhea, urinary tract infection, and various skin diseases (Gajalakshmi, Vijayalakshmi & Rajeswari, 2012). Additionally, the leaves, roots, and seeds of soursop have been reported to demonstrate significant insecticidal properties (Tattersfield, 1940). It has also been documented to possess both hypoglycemic and antioxidant properties without any adverse effects (Lenk et al., 1992). The leaves act also as molluscicidal and anti-parasitical agents (De S. Luna et al., 2005). Extracts from the roots, leaves, and stem are used to make tea and other solutions for patients (Padma, Chansouria & Khosa, 2009). The leaves can be crushed along with raw fruit from the plant and mixed with olive oil to treat various skin disorders, such as rashes, boils, and sores (Padma et al., 2001; Vijayameena et al., 2013). The plant has also been reported to exhibit anti-inflammatory and analgesic effects (Lans, 2006; Roslida et al., 2010; Sarah, Mustafa & Rehab, 2015).

Considering the widespread traditional use of this plant among local communities in Nigeria, it is pertinent to provide scientific support for its application. There is a dearth of such information in some communities, particularly in Ekiti State, where the plants is voraciously exploited for herbal medicinal purposes. Hence, this study was carried out to add to the existing lean body of knowledge on the phytochemical composition as well as antimicrobial properties of the leaves, stem, and bark of *A. muricata* plant on some human pathogens of public health concern.

## Materials and methods

### Sources of plants for extraction

The fresh leaves, stems, and bark of soursop (*Annona muricata*) (Figure 1) were acquired from a market in Ado-Ekiti, Ekiti State, Nigeria, and transported to the laboratory. These parts of the plant were identified at the herbarium unit of the Plant Science Department of Ekiti State University, in Ado-Ekiti.

**Figure 1. *Annona muricata* plant (Moghadamtousi et al., 2015)**

#### Source of test microorganisms

The bacteria and fungi used in this study were clinical isolates from the Department of Medical Microbiology, Federal Teaching Hospital, Ado-Ekiti. They included *Staphylococcus aureus*, *Escherchia coli*, *Salmonella typhi*, *Candida albcans*, and *Candida tropicalis.* The bacteria were maintained on nutrient agar slant at 4°C, while the fungi were maintained on the Potato Dextrose agar slant until needed for assay.

#### Preparation of various solvent extracts

The extraction of the leaves, stem, and bark of *Annona muricata* was carried out by the maceration method, using the solvent polarity of, in order, ethyl acetate, n-hexane, and hot water (100°C). The maceration method used was that described by Ginda et al. (Ginda, Niky & Erly, 2014) and Rarassari and Maftuch (Rarassari & Maftuch, 2016). The soursop leaves, stems, and bark were macerated separately with disinfected mortars and pestles. Exactly 100 g each of the coarsely powdered plant parts were placed in stoppered containers containing 250 ml of solvent (ethyl acetate, n-hexane, or water). They were each labeled appropriately and allowed to stand at room temperature for three days with frequent agitation, until the soluble matter was dissolved. The mixture was strained, the "marc" (the damp solid materials) pressed, and the liquids clarified by filtration after standing (Sukhdev et al., 2008; Sasidharan et al., 2011). These extracts were then concentrated using a rotary evaporator with the temperature not exceeding 40°C until the concentrated extracts were obtained (Rarassari & Maftuch, 2016).

#### Phytochemical screening

Qualitative tests were carried out on the crude solvent extracts for alkaloids, flavonoids, carbohydrates, glycosides, saponins, tannins, terpenoids, proteins, and anthraquinone (Harborne, 1973). These tests were carried out at the Federal University of Technology, Akure, Ondo State, Nigeria, as described below:

#### Test for alkaloids

Exactly 5 ml of the extract was diluted with sulphuric acid to make it acidic. Mayer's regent was added to the acidic extract, a white precipitate indicating the presence of alkaloids, as a positive result.

#### Test for saponins

Exactly 20 ml of the extract was evaporated to dryness and the extract dissolved in 3 ml of chloroform, the filtrate treated with 3 drops of a mixture of concentrated sulphuric acid and acetic anhydride, and a colour of different shade was observed, indicating a positive test for saponins.

#### Test for steroids and terpenes

Five milliliters (5 ml) of the extract was divided into 2 equal parts and evaporated to dryness and the extract dissolved in 3 ml of chloroform. The filtrate was then treated with 3 drops of a mixture of concentrated sulphuric acidic and acetic anhydride. Colors of different shades were observed indicating a positive test. The second portion of the extract was heated with hot acetic anhydride, allowed to cool and six drops of concentrated sulphuric acid added, and a blue-green color was observed, indicating terpenes.

#### Test for tannins and phenols

Exactly 3 ml of extract was treated with 5% ferric chloride solution; a green to blue color was observed indicating a positive test for tannins. Similarly, 3 ml of extract was added to 3 ml of lead acetate solution and a white precipitate occurred, indicating tannins and phenols.

#### Test for proteins

Exactly 1 ml of 4% sodium hydroxide and 1% dilute copper sulphate was added to 5 ml of the extract, and a red solution confirmed proteins. Additionally, a xanthoprotein test was also done by adding 3 ml of extract to 1 ml of concentrated sulphuric acid. The presence of white precipitate which turned to yellow on boiling, and orange on addition of 1 ml ammonium hydroxide, indicated the presence of proteins.

#### Test for carbohydrates

To 2 ml of the extract, 2–3 drops of alpha naphthalene solution in alcohol were added, the solution shaken for 2 minutes, and 1 ml of concentrated sulphuric acid added slowly from the side of the test tube, until it gave a deep purple color at the junction of two layers, indicating the presence of carbohydrate. Adding Benedict's reagent to the extract, it

yielded a yellow to brown precipitate after boiling in a water bath.

#### Test for glycosides

Into 2 ml of extract, 1 ml of pyridine and 1 ml of sodium nitro-prusside were added. A red color indicated the presence of cardiac glycosides.

#### Keller-killiani test

To a test tube containing 2 ml of extract, 1 ml of glacial acetic acid was added with 3 drops of 5% ferric chloride and concentrated sulphuric acid, and the disappearance of the reddish brown color at the junction of the two layers and bluish green in upper layer indicated the presence of cardiac glycosides.

#### Test for flavonoids

Into 2 g of dry extract, 5 ml of ethanol, 5 drops of hydrochloric acid, and 0.5 g of magnesium were added; a pink color indicated the presence of flavonoids.

#### The preparation of extract concentration for antibacterial application

About 3 g each of the concentrated aqueous, ethyl acetate, and n-hexane extracts were dissolved separately in dimethyl sulfoxide (DMSO) until 10 ml of volume was obtained of concentrate from the extract of 300 mg/ml. The dilution was made in order to obtain extracts with concentrations of 5 mg/ml, 10 mg/ml, 50 mg/ml, 150 mg/ml, and 300 mg/ml.

#### Antimicrobial activity assay

The test bacterial innocula (*S. aureus*, *E. coli*, and *S. typhi*) were prepared from an overnight culture of nutrient agar slant. The bacterial cultures were directly suspended in sterile Mueller Hinton broth (oxoid) and the suspension adjusted to the 0.5 Macfarland turbidity standard ($10^5$ cells/ml) needed for the experiment. The fungi innocula were prepared directly using Sabouraud dextrose broth. The already dried Mueller Hinton plate was inoculated with test bacteria using sterile swabs by rotating the plate 3 times between each smear and leaving to dry for 10 minutes at ambient temperature before wells were made. This was repeated for the fungi using a Sabouraud dextrose plate. Exactly 20 µl of each extract concentration (300 mg/ml, 150 mg/ml, 50 mg/ml, 10 mg/ml, and 5 mg/ml (w/v)) was introduced into the wells on already inoculated culture plates with the test bacterial and fungal isolates. These were incubated at 37°C for 24 hours for the bacteria and 48 hours for the fungi. After incubation, each extract's zone of inhibition was noted for each isolate. All tests were done in triplicate. The reference antimicrobial agent, ampicillin, was used as a positive control, while DMSO was used as a negative control. The diameters of zones of inhibition were measured in mm using a Vernier caliper (Vijayameena et al., 2013).

## Results

The phytochemical compositions of extracts of *A. muricata* showed that the leaf, stem, and fruit possess mainly tannins, flavonoids, saponins, reducing sugars, carbohydrates, alkaloids, steroids, proteins, nitrate ions, and starch (Table 1). They also show that only the leaves of *Annona muricata* possess glycosides and there are no phlobatannins and anthraquinone in the leaves, stems, or fruits.

The antimicrobial activities of n-hexane leaf extract of *Annona muricata* on the selected clinical isolates are presented in Table 2. The n-hexane leaf extract showed zone of inhibition diameters of 21 mm against *S. aureus* at 300 mg/ml and 18 mm against *E. coli* at 150 mg/ml. Similarly, the same extract showed 15 mm and 8 mm against *C. tropicalis* at concentrations of 300 mg/ml and 150 mg/ml respectively. Similarly, Tables 3 and 4 show that n-hexane stem extract showed 22 mm and 15 mm against *S. aureus* and *E. coli* respectively.

The ethyl leaf extract at concentrations of 300 mg/ml showed zones of inhibition of 12 mm and 13 mm against *E. coli* and *S. aureus* respectively, while at concentrations of 150 mg/ml, 8 mm was recorded against *E. coli* and *S. aureus* (Table 5). *E. coli* and *S. aureus* showed zones of inhibition of 14 mm and 20 mm respectively at 300 mg/ml (Table 6), and 15 mm and 8 mm respectively at 300 mg/ml (Table 7).

The hot water extracts of *A. muricata* also exhibited antimicrobial effects recorded in Tables 8, 9, and 10. They show diameters of inhibition of 10 mm at 150 mg/ml and 18 mm at 300 mg/ml against *E. coli*. *Candida albicans* and *Candida tropicalis* displayed 5 mm and 8 mm respectively at 300 mg/ml. (Table 8). *E. coli* and *S. aureus* showed zones of inhibition of 10 mm and 22 mm respectively at 300 mg/ml (Table 9), and 2 mm each at 300 mg/ml (Table 10).

**Table 1. Phytochemical constituents of leaf, stem, and fruit extracts of soursop**

| Phytochemical constituents | Leaf | Stem | Fruit |
|---|---|---|---|
| Tannins | + | + | + |
| Flavonoids | + | + | + |
| Glycosides | + | – | – |
| Saponins | + | + | + |
| Phlobatannins | – | – | – |
| Reducing sugar | + | + | + |
| Carbohydrate | + | + | + |
| Alkaloids | + | + | + |
| Steriods | + | + | + |
| Protein | + | + | + |
| Nitrate ion | + | + | + |
| Starch | + | + | + |
| Anthraquinone | – | – | – |

+ present; – absent

**Table 2. Antimicrobial activities of n-hexane leaf extract of *Annona muricata* (mm)**

| Clinical Isolates | 5 mg/ml | 10 mg/ml | 50 mg/ml | 150 mg/ml | 300 mg/ml |
|---|---|---|---|---|---|
| *Escherichia coli* | 0 | 0 | 0 | 18 | 20 |
| *Staphylococcus aureus* | 0 | 0 | 2 | 15 | 21 |
| *Salmonella typhi* | 0 | 0 | 0 | 2 | 5 |
| *Canadia albicans* | 0 | 0 | 0 | 8 | 10 |
| *Canadia tropicalis* | 0 | 0 | 5 | 8 | 15 |

**Table 3. Antimicrobial activities of n-hexane stem extract of *Annona muricata***

| Clinical Isolates | 5 mg/ml | 10 mg/ml | 50 mg/ml | 150 mg/ml | 300 mg/ml |
|---|---|---|---|---|---|
| *Escherichia coli* | 0 | 0 | 0 | 10 | 15 |
| *Staphylococcus aureus* | 0 | 0 | 0 | 6 | 22 |
| *Salmonella typhi* | 0 | 0 | 0 | 0 | 4 |
| *Canadida albicans* | 0 | 0 | 0 | 0 | 0 |
| *Canadida tropicalis* | 0 | 0 | 0 | 0 | 0 |

**Table 4. Antimicrobial activities of n-hexane fruit extract of *Annona muricata***

| Microorganism | 5 mg/ml | 10 mg/ml | 50 mg/ml | 150 mg/ml | 300 mg/ml |
|---|---|---|---|---|---|
| *Escherichia coli* | 0 | 0 | 0 | 10 | 15 |
| *Staphylococcus aureus* | 0 | 0 | 0 | 6 | 22 |
| *Salmonella typhi* | 0 | 0 | 0 | 0 | 4 |
| *Canadida albicans* | 0 | 0 | 0 | 0 | 0 |
| *Candida tropicalis* | 0 | 0 | 0 | 0 | 0 |

**Table 5. Antimicrobial activities of Ethyl acetate leaf extract of *Annona muricata***

| Microorganism | 5 mg/ml | 10 mg/ml | 50 mg/ml | 150 mg/ml | 300 mg/ml |
|---|---|---|---|---|---|
| *Escherichia coli* | 0 | 0 | 0 | 8 | 12 |
| *Staphylococcus aureus* | 0 | 0 | 4 | 8 | 13 |
| *Salmonella typhi* | 0 | 0 | 0 | 0 | 5 |
| *Candida albicans* | 0 | 0 | 0 | 4 | 8 |
| *Candida tropicalis* | 0 | 0 | 3 | 6 | 10 |

**Table 6. Antimicrobial activities of Ethyl acetate extract stem extract of *Annona muricata***

| Clinical Isolate | 5 mg/ml | 10 mg/ml | 50 mg/ml | 150 mg/ml | 300 mg/ml |
|---|---|---|---|---|---|
| *Escherichia coli* | 0 | 0 | 5 | 8 | 14 |
| *Staphylococcus aureus* | 0 | 2 | 8 | 15 | 20 |
| *Salmonella typhi* | 0 | 0 | 0 | 0 | 0 |
| *Candida albicans* | 0 | 0 | 0 | 0 | 4 |
| *Candida tropicalis* | 0 | 0 | 0 | 0 | 5 |

**Table 7. Antimicrobial activities of Ethyl acetate extract fruit extract of *Annona muricata***

| Clinical Isolate | 5 mg/ml | 10 mg/ml | 50 mg/ml | 150 mg/ml | 300 mg/ml |
|---|---|---|---|---|---|
| *Escherichia coli* | 0 | 0 | 0 | 10 | 15 |
| *Staphylococcus aureus* | 0 | 0 | 0 | 0 | 8 |
| *Salmonella typhi* | 0 | 0 | 0 | 0 | 0 |
| *Candida albicans* | 0 | 0 | 0 | 0 | 0 |
| *Candida tropicalis* | 0 | 0 | 0 | 0 | 0 |

**Table 8. Antimicrobial activities of hot water leaf extract of *Annona muricata***

| Clinical Isolates | 5 mg/ml | 10 mg/ml | 50 mg/ml | 150 mg/ml | 300 mg/ml |
|---|---|---|---|---|---|
| *Escherichia coli* | 0 | 0 | 0 | 10 | 18 |
| *Staphylococcus aureus* | 0 | 0 | 0 | 5 | 20 |
| *Salmonella typhi* | 0 | 0 | 0 | 2 | 4 |
| *Candida albicans* | 0 | 0 | 0 | 0 | 5 |
| *Candida tropicalis* | 0 | 0 | 0 | 4 | 8 |

**Table 9. Antimicrobial activities of hot water stem extract of *Annona muricata***

| Clinical Isolates | 5 mg/ml | 10 mg/ml | 50 mg/ml | 150 mg/ml | 300 mg/ml |
|---|---|---|---|---|---|
| *Escherichia coli* | 0 | 0 | 0 | 0 | 10 |
| *Staphylococcus aureus* | 0 | 0 | 0 | 6 | 22 |
| *Salmonella typhi* | 0 | 0 | 0 | 0 | 0 |
| *Candida albicans* | 0 | 0 | 0 | 0 | 0 |
| *Candida tropicalis* | 0 | 0 | 0 | 0 | 0 |

**Table 10. Antimicrobial activities of hot water fruit extract of *Annona muricata***

| Clinical Isolates | 5 mg/ml | 10 mg/ml | 50 mg/ml | 150 mg/ml | 300 mg/ml |
|---|---|---|---|---|---|
| *Escherichia coli* | 0 | 0 | 0 | 0 | 2 |
| *Staphylococcus aureus* | 0 | 0 | 0 | 0 | 2 |
| *Salmonella typhi* | 0 | 0 | 0 | 0 | 0 |
| *Candida albicans* | 0 | 0 | 0 | 0 | 0 |
| *Candida tropicalis* | 0 | 0 | 0 | 0 | 0 |

## Discussion

The phytochemical constituents of leaf, stem, and fruit extracts of *Annona muricata* revealed tannins, flavonoids, saponins, reducing sugars, carbohydrates, alkaloids, steroids, proteins, and starch. Previous studies have shown that the antimicrobial

properties of plants are due to tannins, alkaloids, saponins, flavonoids, sterol, triterpenes, and reducing sugars (Pathak et al., 2010; Ogu & Amiebenomo, 2012; Ogu et al., 2012; Ogu, Ezeadila & Ehiobu, 2013). Thus, the antibacterial activities of the leaf, stem, and bark extracts observed in this study are most likely due to the presence of one or more of these bioactive principles in the extract. The leaves possess more phytochemical components than the stem or fruits. This suggests that that the leaves may be utilized for the strongest beneficial effects on human health. This finding agrees with studies conducted by Edeoga et al. (Edeoga, Okwu & Mbaebie, 2005) and Usunobum and Paulinus (Usunobum & Paulinus, 2015), who report that *Annona muricata* are reservoirs of free radical scavenging molecules, rich in antioxidant activity.

This study tested for and observed antimicrobial activities of n-hexane, ethyl acetate, and aqueous extracts of the leaves, stems, and fruits of soursop on selected clinical isolates. It showed that n-hexane, ethyl acetate, and aqueous extracts of soursop leaves inhibited *E. coli*, *S. aureus*, *S. typhi*, *C. albicans* and *C. tropicalis* at concentrations of 300 mg/ml and 150 mg/ml. There was no sensitivity recorded at lower concentrations against the tested isolates except at the concentration of 50 mg against *S. aureus* and *C. tropicalis.* This corroborates the earlier report of Sarah et al. (Sarah, Mustafa, & Rehab, 2015), of the antibacterial effect of methanolic and aqueous extracts of the leaves of *Annona muricata* against various bacterial strains: *Staphylococcus aureus* ATCC29213, *Escherichia coli* ATCC8739, *Proteus vulgaris* ATCC13315, *Streptococcus pyogenes* ATCC8668, *Bacillus subtilis* ATCC12432, *Salmonella typhi* ATCC23564, and *Klebsiella pneumonia.* This is supported by the previous reports of Lans (2006) who demonstrated that the leaf, bark, root, stem, fruit and seed extracts of *Annona muricata* possess anti-bacterial, antifungal, and anti-malarial properties. Similarly, the n-hexane, ethyl acetate, and aqueous extracts of soursop stems in this study demonstrated antibacterial and antifungal effects against *E. coli*, *S. aureus*, *S. typhi*, *C. albicans*, and *C. tropicalis* at concentrations of 300 mg/ml and 150 mg/ml. Thus, *Annona muricata* extract contains a wide spectrum of activity against a group of bacteria responsible for the most common bacterial diseases. Pathak et al. (2010) also reported that leaf extract of *Annona muricata* is used in the treatment of various bacterial infectious diseases. Thus, the plant possesses an abundance of antibacterial compounds as reported earlier (Moghadamtousi et al., 2015).

Nevertheless, in this study, although the n-hexane extract of the fruit showed antibacterial effects on all the selected bacteria at 300 mg/ml and 150 mg/ml, the *Candida* species were resistant at the same concentrations. This suggests that more of the antibacterial bioactive ingredients were soluble in n-hexane than the antifungal components. The slightly greater antimicrobial activities recorded in this study for leaf extract over stem or bark extracts, suggests that more of the bioactive ingredients are lodged in the leaves, as reported by Ogu et al. (Ogu et al., 2012). This is probably one of the reasons herbal practitioners have almost always recommended using leaf extracts over those of stems or barks in native herbal medicine. This submission is in consonance with the submissions of previous studies (Adeshina, Onujagbe & Onaolapo, 2010; Ogu et al., 2012). The aqueous extract of the stem and leaves showed that hot water leaf extraction resulted in better antibacterial effects than cold extraction, indicating that most of the active agents were expressed by hot rather than cold maceration.

Studies in the past have reported similar finding (Matsushige, Kotake & Takeda, 2012). The findings in this study further support earlier claims that medicinal plants can be used for effective treatments of infectious diseases caused by a variety of microorganisms, and thus should be exploited.

## Conclusions

Many common plant-based foods contain powerful antimicrobial phytochemical substances that can improve human health. The antimicrobial properties demonstrated for different parts and fractions of *Annona muricata* might provide a good alternative to antibiotic drugs in the treatment of some infections. The phytochemicals found in this study could also offer significant protections to consumers against many diet related diseases, including cancer, because of the presence of antioxidants. Therefore this study suggests that every part of the *Annona muricata* can be used for numerous health benefits and should be prepared and consumed in an appropriate manner in order to confer the most health benefits possible.

*Annona muricata* (soursop) is an essential medicinal plant which has been reported to promote the general health of human beings. Its potential as a source of new drugs cannot be over-emphasized. Therefore, proper and adequate use of the plant will be a welcome development. Also, it would probably be beneficial to incorporate some of the active

substances into foods and drinks, and finally, the molecular study of the plant will provide more vital information about its potential as a good drug alternative.

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# Antimicrobial and Phytochemical Analysis of Methanolic and Aqueous Extract of *Annona muricata* (Leaf and Fruit)

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**A B S T R A C T**

*Annona muricata* is a species of the genus *Annona* of the custard apple tree family, Annonaceae, which has edible fruit. The aim of this study was to carryout for check the Antibacterial, Antifungal and Phytochemical activity from methanolic and aqueous extract of leaf and fruit of *Annona muricata*. An antibacterial activity of *Annona muricata* was evaluated on Pathogenic bacteria (*Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa, Salmonella typhi, Klebsiella pneumoniae, Escherichia coli* and *Proteus mirabilis*) by Well diffusion method. Antifungal activity of *Annona muricata* was evaluated on *Cryptococcus neoformans* and *Candida albicans*. Phytochemical analysis was done by using standard methods. Phytochemical screening was used to determine the presence of Alkaloids, Flavonoids, Carbohydrates, Glycosides, Proteins, Amino acids, Saponins, Tannins, Terpenoids in Methanolic and Aqueous extract of *Annona muricata*. These findings support the traditional use of *Annona muricata* in varies disorders.

## Introduction

Plants are one of the most important sources of medicines for treating illnesses since the beginning of human civilization (Jyothi *et al.*, 2011). In recent times, and due to historical, cultural, and other reasons, folk medicine has taken an important place, especially in developing countries, where health services are limited. The study on the medicinal plants is essential to promote the proper use of herbal medicine in order to determine their potential as a source for the new drugs (Gajalakshmi *et al.*, 2012).

Ayurvedic medicine uses all of these either in diet or as a medicine. Some of these medicinal plants like Tulsi (*Ocimum sanctum*), Haridra (*Curcuma longa*), sarpagandha (*Rauvolfia serpentia*) and Ghritkumari (*Aleo barbadensis*) have been featured on Indian postage stamps. Among this one of the important medicinal plants which show many medicinal properties is *Annona muricata* (In Tamil, Mullu seetha). *Annona muricata* is a member of the family of custard apple tree called Annonaceae and

species of the genus *Annona* known mostly for its edible fruit Annona. It is a small upright evergreen tree 5-6 meter height with large, glossy, dark green leaves. It produce a large heart shaped, edible fruit, 5-20 cm in diameter, yellow green in colour and has white flesh inside. *Annona muricata* produce fruit that are usually called soursop due to its slightly acidic taste when ripe (Hutchinson *et al.*, 2011). The fruit is juicy, acidic, whitish and aromatic with abundant seeds. The average weight of 1000 fresh seeds is 479g and has an average oil content of 24% (Hutchings, 2006). The creamy and delectable flesh of fresh fruit consists of 80% of water, 1% protein, 18% carbohydrate and fair amount of Vitamin B, B2 and C and Potassium and dietary fiber. Annonaceous acetogeneins are powerful phytochemicals found in the Graviola plant (*Annona muricata*), which are found only in Annonaceae family. These chemicals in general have been documented with Antitumor, Antiparasitic, Insecticidal and Anti-microbial activities. These acetogeneins are superb inhibitors of enzyme processes that are found only in the membranes of cancerous tumour cells.

The leaves and stems of soursop show active cytotoxicity against cancer cells, due to bioactive compounds called acetogenins. These acetogenins are non-toxic to normal cells, but are highly toxic to cancer cells (Oberlies *et al.,* 1995). Acetogenins from the soursop plant are usually extracted using ethanol as the organic solvent (Zeng *et al.,* 1996). These compounds, collectively, have shown antitumor, parasiticidal, pesticidal, and antimicrobial activities (McLaughlin, 2008). The cytotoxicity of annonaceous acetogenins is due to the depletion of ATP levels *via* the inhibition of the NADP-ubiquinone oxidoreductase (Complex I) of the mitochondrial electron transport system (Zafra-Polo *et al.,* 1996). The lactone ring present in acetogenin molecules plays an important role in the anticancer mechanism, present (Kreuger *et al.,* 2012). Annonacin is known as the most prevalent acetogenin present in soursop leaves (Champy *et al.,* 2004; Yuan *et al.,* 2003), however, the method for separation of annonacin from soursop leaves is not widely reported. More reports are available on various methods to obtain new acetogenins from soursop leaves (Kim *et al.,* 1998; Zeng *et al.,* 1996).

The objective of this research work is to check the antimicrobial activity of *Annona muricata* against pathogenic organisms like *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis, Klepsiella pneumonia* and *Salmonella typhi.* Antifungal activity was checked against *Candida albicans* and *Cryptococcus neoformans.* And to screen the aqueous and methanolic extract of *Annona muricata* for their biologically active chemicals, with a view to provide a scientific basis for use of the leaves for prevention and treatment of diseases.

## Materials and Methods

### Sample collection and authentication

*A.muricata* L. Leaves and fresh ripe fruits (5 kg) were collected locally at Gudalur, Ooty in the month of July 2016. Leaves and fruits were authenticated (BSI/SRC/5/23/ 2016/Tech/1223) by the authority of the botanical survey of India (BSI), Tamil Nadu Agricultural University, Coimbatore.

### Sample preparation

*A.muricata* leaves were washed thoroughly under running tap water, air dried and homogenized to fine powder and stored in sterile air tight container for the

experimental work. *A.muricata* fruits were washed in tap water Seeds and pericarps were carefully removed and pulp was lyophilized for further use.

## Preparation of Methanolic and Aqueous extracts

Air dried powder of leaves and lyophilized fruit pulp was extracted by using Soxhlet apparatus. 10 g of *A.muricata* leaf and fruit powder was taken in a paper cone and placed into soxhlet apparatus. 100 ml of solvent (methanol and water) was taken in the round bottom flask attached to this setup. Then the whole setup was placed on the heating mantle. The temperature was setup to 65-80$^{o}$C solvents get vaporized and rises up to the condenses back in to the liquid and falls in to the plant sample in the cone and extract certain compounds falls in to the round bottom flask.

Methanol extract of leaf (dark green color) and fruit (yellowish brown) was evaporated to dryness at low temperature, by using rotary evaporator. And aqueous extract of leaf (brown color) and fruit (light brown) was evaporated by hot plate method (Satyanarayana, 2006). Concentrated extracts were dissolved in DMSO for antimicrobial studies.

## Microbial cultures

Microorganisms *Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa, Salmonella typhi, Klebsiella pneumoniae, Escherichia coli, Proteus mirabilis, Cryptococcus neoformans* and *Candida albicans* were obtained from KMCH Hospital, Coimbatore, Tamilnadu. And the cultures were further conformed by staining and biochemical methods. Antimicrobial activity was examined for aqueous and methanol extract from the fruit and leaves of *Annona muricata.*

## Antimicrobial activity by well diffusion method

Molten Mueller Hinton agar was prepared and poured into petri dishes and the bacterial culture was swabbed on the surface of agar to check the antibacterial activity. And a well was made by using 6mm well cutter. Aqueous and methanolic extracts of fruit and leaves of *Annona muricata* was added into well in the concentration of 50µl, 100µl and 200µl respectively. For each bacterial strain positive and negative controls were maintained. Antibacterial activity of the extract was determined by measuring the diameter of zone of inhibition.

To check the antifungal activity Sabouraud dextrose agar was prepared and poured into petri dishes and the fungal culture was swabbed on the surface of agar. A well was made by using 6mm well cutter.

Aqueous and methanolic extracts of fruit and leaves of *Annona muricata* was added into well in the concentration of 50µl, 100µl and 200µl respectively. For each fungal culture positive and negative controls were maintained. Antifungal activity of the extract was determined by measuring the diameter of zone of inhibition.

## Phytochemical screening

Phytochemical examinations were carried out for all the extracts as per the standard methods. (Roopashree *et al.,* 2008; Obasi *et al.,* 2010).

## Results and Discussion

## Antibacterial activity

Antibacterial activity for ethanolic and aqueous extract of *Annona muricata* was seen against to the several organisms namely

*Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa, Salmonella typhi, Klebsiella pneumoniae, Escherichia coli* and *Proteus mirabilis.*

The methanol and aqueous leaf extract showed maximum activity against *Streptococcus pyogenes.* And the methanol and aqueous fruit extract showed maximum activity against *Staphylococcus aureus* and *Salmonella typhi* as shown in table 1 and 2.

**Antifungal activity**

Antifungal activity for ethanolic and aqueous extract of *Annona muricata* was seen against *Candida albicans* and *Cryptococcus neoformans.* The methanol and aqueous leaf extract showed maximum activity against *Cryptococcus neoformans.* And the methanol and aqueous fruit extract showed maximum activity against *Candida albicans* as shown in Table 3 and 4.

**Table.1** Antibacterial activity for methanolic and aqueous leaf extract of *Annona muricata*

| Test organisms | Concentration of methanol extract(µl) | | | Concentration of aqueous extract(µl) | | | Positive control (Streptomycin 0.1mg/ml) | Negative control (DMSO) |
|---|---|---|---|---|---|---|---|---|
| | 50 | 100 | 200 | 50 | 100 | 200 | | |
| | Diameter of inhibition (mm) | | | | | | Diameter of inhibition(mm) | |
| *Staphylococcus aureus* | 14 | 20 | 22 | 10 | 12 | 15 | 18 | - |
| *Streptococcus pyogenes* | 18 | 20 | 24 | 11 | 14 | 18 | 20 | - |
| *Pseudomonas aeruginosa* | 15 | 21 | 22 | 10 | 13 | 16 | 17 | - |
| *Klebsiella pneumonia* | 14 | 22 | 23 | 13 | 15 | 18 | 20 | - |
| *Proteus mirabilis* | 13 | 19 | 21 | - | - | 13 | 15 | - |
| *Escherichia coli* | 20 | 22 | 24 | 10 | 13 | 15 | 20 | - |
| *Salmonella typhi* | 18 | 20 | 22 | 15 | 18 | 20 | 20 | - |

**Table.2** Antibacterial activity for methanolic and aqueous fruit extract of *Annona muricata*

| Test organisms | Concentration of methanol extract(µl) | | | Concentration of aqueous extract(µl) | | | Positive control (Streptomycin 0.1mg/ml) | Negative control (DMSO) |
|---|---|---|---|---|---|---|---|---|
| | 50 | 100 | 200 | 50 | 100 | 200 | | |
| | Diameter of inhibition (mm) | | | | | | Diameter of inhibition(mm) | |
| *Staphylococcus aureus* | 13 | 18 | 24 | 18 | 20 | 22 | 18 | - |
| *Streptococcus pyogenes* | - | 15 | 20 | - | 11 | 17 | 20 | - |
| *Pseudomonas aeruginosa* | 11 | 15 | 18 | - | 13 | 18 | 17 | - |
| *Klebsiella pneumonia* | - | 18 | 22 | - | - | 14 | 20 | - |
| *Proteus mirabilis* | - | - | 18 | - | - | 15 | 15 | - |
| *Escherichia coli* | - | 18 | 22 | - | - | 14 | 20 | - |
| *Salmonella typhi* | 18 | 22 | 28 | 15 | 18 | 26 | 20 | - |

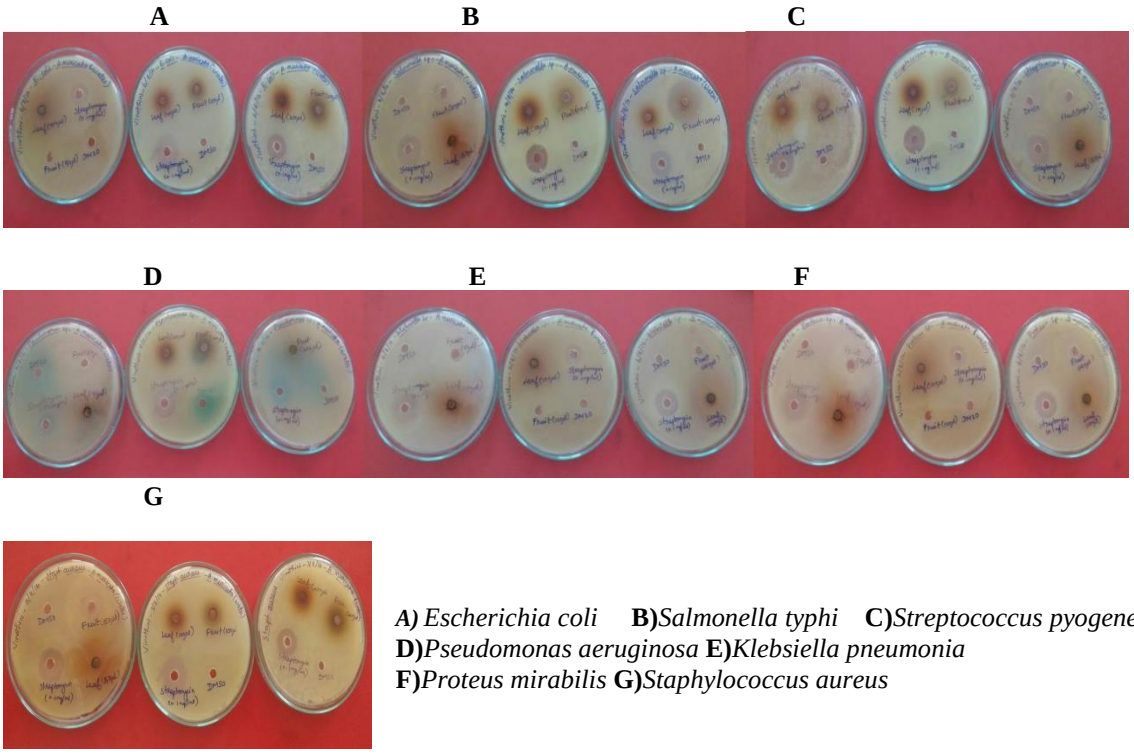
**Table.3** Antifungal activity for methanolic and aqueous leaf extract of *Annona muricata*

| Test organisms | Concentration of methanol extract(µl) | | | Concentration of aqueous extract(µl) | | | Positive control (Amphotericin B 0.1mg/ml) | Negative control (DMSO) |
|---|---|---|---|---|---|---|---|---|
| | 50 | 100 | 200 | 50 | 100 | 200 | | |
| | Diameter of inhibition (mm) | | | | | | Diameter of inhibition(mm) | |
| *Candida albicans* | - | 14 | 16 | 14 | 16 | 18 | 20 | - |
| *Cryptococcus neoformans* | 18 | 20 | 24 | 17 | 19 | 21 | 18 | - |

**Table.4** Antifungal activity for methanolic and aqueous fruit extract of *Annona muricata*

| Test organisms | Concentration of methanol extract(µl) | | | Concentration of aqueous extract(µl) | | | Positive control (Amphotericin B 0.1mg/ml) | Negative control (DMSO) |
|---|---|---|---|---|---|---|---|---|
| | 50 | 100 | 200 | 50 | 100 | 200 | | |
| | Diameter of inhibition (mm) | | | | | | Diameter of inhibition(mm) | |
| *Candida albicans* | 18 | 20 | 23 | 18 | 20 | 22 | 18 | - |
| *Cryptococcus neoformans* | 18 | 20 | 22 | 15 | 17 | 18 | 20 | - |

**Fig.1** Antibacterial activity for Aqueous extract of *Annona muricata*

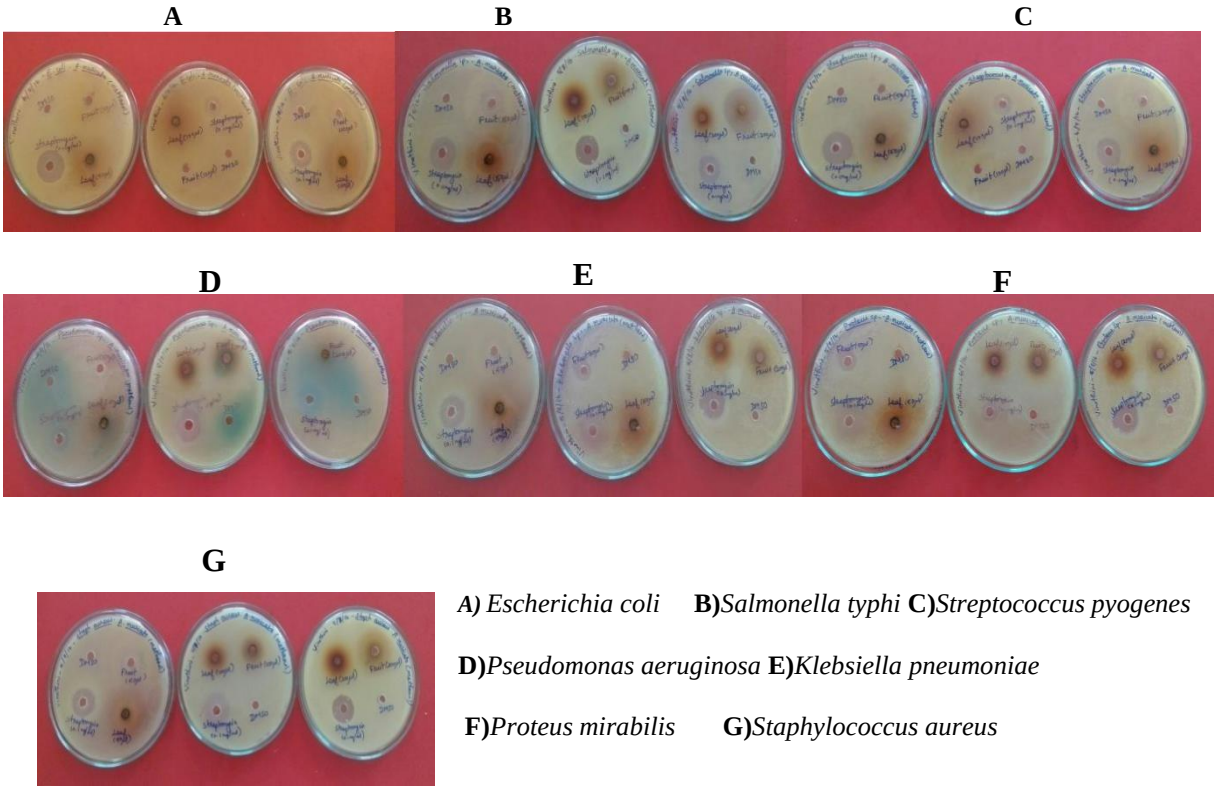



**A)** *Escherichia coli* **B)***Salmonella typhi* **C)***Streptococcus pyogenes*
**D)***Pseudomonas aeruginosa* **E)***Klebsiella pneumonia*
**F)***Proteus mirabilis* **G)***Staphylococcus aureus*

**Table.5** Qualitative phytochemical screening of aqueous and methanol extract of *Annona muricata*

| S.No | Test | Aqueous extract | | Methanol extract | |
|---|---|---|---|---|---|
| | | **Leaf** | **Fruit** | **Leaf** | **Fruit** |
| 1. | Test for alkaloids | | | | |
| | a)Mayer's test | + | + | + | + |
| | b)Wagner's test | + | - | + | - |
| | c)Hager's test | + | + | + | + |
| **2.** | Test for flavonoids | | | | |
| | a)Alkaline reagent test | + | + | + | + |
| | b)Lead acetate test | + | - | + | + |
| **3.** | Test for carbohydrates | | | | |
| | a)Benedict's test | + | + | + | + |
| | b)Molisch's test | + | + | + | + |
| | c)Fehling's Test | + | + | + | + |
| **4.** | Test for glycosides | | | | |
| | a)Borntrager's test | + | + | + | + |
| | b)Legal's test | + | + | + | + |
| **5.** | Test for saponin | | | | |
| | a)Froth test | + | + | + | + |
| | b)Foam test | + | + | + | + |
| **6.** | Test for phenols | | | | |
| | a)Ferric chloride test | - | - | + | + |
| **7.** | Test for tannins | | | | |
| | a)Gelatin test | + | - | + | - |
| **8.** | Test for protein and amino acids | | | | |
| | a)Xanthoproteic test | + | + | + | + |
| | b)Ninhydrin test | + | + | + | + |
| **9.** | Test for terpenoids | | | | |
| | a)Salkowski test | + | - | + | - |
| | b)Copper acetate test | + | + | + | + |
| **10.** | Test for anthraquinone | | | | |
| | a)Ammonia test | - | - | - | - |

**Fig.2** Antibacterial activity for methanol extract of *Annona muricata*



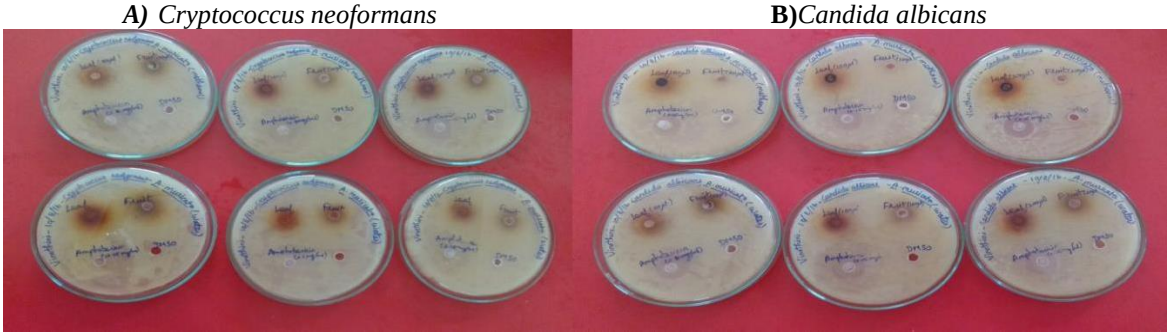

***A)*** *Escherichia coli* **B)***Salmonella typhi* **C)***Streptococcus pyogenes*

**D)***Pseudomonas aeruginosa* **E)***Klebsiella pneumoniae*

**F)***Proteus mirabilis* **G)***Staphylococcus aureus*

**Fig.3** Antifungal activity for methanol and aqueous extract of *Annona muricata*

***A)*** *Cryptococcus neoformans* **B)***Candida albicans*



## Phytochemical screening

The present study reveals that *Annona muricata* plant shows the presence of phytochemical constituents like alkaloids, carbohydrates, glycosides, proteins, aminoacids, saponins, tannins, terpenoids and anthraquinones in methanol and aqueous leaf and fruit extract of *Annona muricata* as shown in Table 5.

Antimicrobial activity of methanol and aqueous extract of *Annona mricata* (fruit and leaf) was determined by well diffusion method (Abubacker *et al.,* 2012). Methanol extract of *Annona muricata* showed maximum antimicrobial activity than aqueous extract. Results of the phytochemical screening of *Annona muricata* methanolic leaf and fruit extract showed the absence of anthraquinone while

flavonoids, saponins, tannins, phenols, alkaloids, terpenoids, protein, amino acids and glycosides were present. And *Annona muricata* aqueous leaf and fruit extract showed the absence of phenol and anthraquinone. (Roopashree *et al.,* 2008; Obasi *et al.,* 2010).These phytochemicals exhibit various pharmacological and biochemical actions when ingested by animals. The comparative antimicrobial activity between methanolic and aqueous extracts of *Annona muricata* and the standard antibiotic streptomycin and amphotericin B revealed that the methanolic extract showed significant antimicrobial efficacy and could compete with the standard antibiotic, streptomycin and amphotericin B. This study discovered that *Annona muricata* extract possess a broad spectrum of activity against a panel of microbial responsible for the most common bacterial and fungal diseases.

In conclusion, the present study demonstrates that *Annona muricata* has antibacterial and antifungal activity against various test organisms like *Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa, Salmonella typhi, Klebsiella pneumoniae, Escherichia coli, Proteus sp., lactobacillus sp., Cryptococcus neoformans* and *Candida albicans.* And this study shows that *Annona muricata* is a good source for various phytochemicals like alkaloids, flavonoids, carbohydrates, glycosides, saponins, tannins, terpenoids, proteins and anthraquinone. All these preliminary reports form a primary platform for further phytochemical and pharmacological studies.

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# Susceptibility Patterns of Some Enteric Bacteria to Crude and Purified Bark Extracts of *Annona muricata* L.

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***Authors' contributions***

*This work was carried out in collaboration between both authors. Author EOD designed the study and wrote the protocol. Author KYA performed the statistical analysis, wrote the first draft of the manuscript, managed the analyses of the study and managed the literature searches. Author EOD guided in the entire research and edited the final draft of the manuscript. Both authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** This research was carried out to evaluate the susceptibility pattern of some enteric bacteria to crude and purified extracts of *Annona muricata* bark.
**Study Design:** Experimental design.
**Place and Duration of Study:** Department of Microbiology, Federal University of Technology, Akure, Ondo State, Nigeria. Between January, 2019 and May, 2019.
**Methodology:** Extraction of bioactive components of bark was done by maceration and phytochemical screening was carried out on the bark extracts to determine the bioactive components present. The bacteria isolates were subjected to antibiotic sensitivity test using standard methods while the antibacterial activity of the plant extracts on human enteric bacteria was determined using agar well dilution method. *A. muricata* bark extracts were purified using column chromatography method. The minimum inhibitory and minimum bactericidal concentrations (MIC/MBC) of the extracts were performed using tube dilution technique.

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**Results:** The quantitative phytochemical screening for bark extract revealed that glycosides (7.06±0.04, 34.67±0.02 and 19.35±0.01) extracted with aqueous, ethanol and methanol respectively is the most abundant phytochemical constituents. The antibacterial activities of the bark extracts revealed that aqueous showed no inhibition to none while ethanol and methanol inhibited all the test organisms. The highest value of minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) for both ethanol and methanol bark extracts was 50 mg/ml and 100 mg/ml respectively.
**Conclusion:** This research revealed that *A. muricata* bark extracts possesses antibacterial activity against human enteric bacteria isolates used in this study. The purified extracts of *A. muricata* bark showed higher zones of inhibition which indicates that it can compete well with standard antibiotics and it may also serve as a substitute to the commercially available antibiotics that can be used for the treatment of infections caused by enteric bacteria.

*Keywords: Antibacterial activity; ethanol extract; Annona muricata bark; phytochemical constituents; enteric bacteria.*

## 1. INTRODUCTION

Different plants and their parts are used all over the world for various purposes. Study reported that plants have an efficient constituents which are mostly used as antioxidant, antibacterial, antifungal, antiulcer, anti-inflammatory, antiviral and anticancer agents [1].

*Annona muricata* (L.) is referred to as graviola, guanabana or soursop in English-speaking countries and in Nigeria. It belongs to a family called Annonaceae. *A. muricata* is used in traditional medicine in many regions. It is popularly grown across the tropical regions of the world [2]. The plant is known to produce an edible fruit that is green in colour, large, heart-shaped and 15–20 cm in diameter with a white fleshy mesocarp [3]. The plant parts have been used from time immemorial, thus its ethnopharmacological use. Generally, the fruit and its juice are used to combat worms and parasitic organisms, to cool fevers, increase breast milk production after birth, and as an astringent for diarrhea and dysentery [1]. The fresh leaves when crushed are applied on skin eruption for quick healing. The leaf or bark tea or combination of both is used as a sedative and heart tonic by the indigenes of Guyana [3]. Studies have revealed that the barks, fruits, leaves and seeds of *A. muricata* consist of biological and pharmacological activities such as antimicrobial, cytotoxic, anti-parasitic and pesticidal activities [4].

Enteric bacteria are bacteria that have the ability of causing enteric diseases. Enteric bacteria pathogens are one of the major causes of food borne gastroenteritis in humans and remain an important health problem worldwide [5]. Enteric bacteria are microbes that reside in the guts of animals and humans. They can cause a mild infection, such as food poisoning or severe community infections like diarrhea [6].

Food borne diseases are important cause of morbidity and mortality worldwide. Most food borne bacterial infections cause self-limiting diarrhea, systemic infection and however, death can occur, particularly in vulnerable groups such as the elderly, people with diminished immunity or infants and young children [5]. Bacteria have accounted for more than 70% of deaths associated with food borne transmission [7].

The emergence and spread of antibiotic resistance continue to be an important global problem particularly in developing countries. The increasing drug resistance is partly due to the frequent mutation of the pathogens and partly because of the overuse or misuse of drugs [8]. Antibiotic resistance in bacteria continues to spread and cause morbidity, mortality, and increase in cost of the treatment of infectious diseases due to treatment failures [9].

It has been documented that *A. muricata* posseses saponins, tannins and glycosides as the major constituents and trace amounts of flavonoids which contribute immensely to the bioactivity of *A. muricata* and also its usage in treating various diseases [10]. However, there is limited research work comparing the antibacterial activity of crude and purified extracts of *A. muricata* against enteric bacteria isolates. Hence, the need to evaluate the susceptibility patterns of some enteric bacteria to crude and purified extracts of *A. muricata* bark.

## 2. MATERIALS AND METHODS

### 2.1 Plant Collection and Identification

Fresh bark of *A. muricata* L. was collected from a garden at Adebayo, Ado Ekiti, Ekiti State. The leaves and bark were identified and authenticated at the Department of Crop, Soil and Pest management, Federal University of Technology, Akure (FUTA).

### 2.2 Extraction of *A. muricata* Bark

The leaf and bark of *A. muricata* were washed with sterile water, air dried for three weeks and pulverized into fine powder. The bark were coarsely powdered using a sterile mortar and pestle and were further pulverized to powder using an electric blender. Fifty grams (50 g) of the powdered bark was soaked into one liter (1L) of cold water, 100% ethanol and methanol. The container of the mixtures were labeled and left covered for 3 days (72 hours) with intermittent agitation followed by sieving with a muslin cloth and filtered using No 1 Whatman filter paper. The filtrates were vaporized to dryness using rotary evaporator and subsequently lyophilized to remove the extracting solvent. The crude extracts obtained was preserved in a sterile container and stored in the refrigerator at 4ºC until when ready for use [1]. The crude extracts which were obtained were sterilized using 0.45 µm millipore membrane filter in order to remove any contaminant that might be present before subsequent use.

The weight of the dried extracts was measured and the percentage recovery was calculated as;

> Percentage recovery = (Weight of extract recovered after extraction×100%) / Initial weight of plant part.

### 2.3 Purification of *A. muricata* Bark Extracts

The plant extracts were purified according to the method described by Atta, et al. [12]. The column was packed with silica gel of 60-120 mesh. During this process of packing the gel, outmost care was taken in other to avoid distortion and cracking of the gel. A 250 ml burette was attached to a retort stand; small piece of glass wool was tucked down lightly to avoid particles from the cotton dropping into the fraction during separation into the burette with the aid of an applicator stick. For the mobile phase 1: 1: 1 proportions of three solvents were used. 100g of silica gel was mixed thoroughly with equal volume of Methanol, Ethanol and Acetone and poured into the burette. A 100 ml of the solvents was used to top the silica gel for it to flow down slowly to allow the proper packing of the column. Two (2 g) grams of the crude extracts was mixed with 5 ml of the solvents and added carefully unto the surface of the column. More solvent was added as the fractions of the extracts were being obtained in small sterile containers.

### 2.4 Phytochemical Screening of *A. muricata* Bark Extracts

The aqueous, ethanol and methanol bark extracts of *A. muricata* were subjected to qualitative and quantitative phytochemical screening for the presence of bioactive constituents such as tannins, phenols, alkaloids, glycosides, anthroquinones, saponins and flavonoids [11].

### 2.5 Collection of Bacterial Isolates

Clinical enteric bacteria isolates (*Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris Pseudomonas aeruginosa, Salmonella typhi* and *Shigella dysenteriae*) were obtained from the stock culture of Ekiti State University Teaching Hospital, Ado Ekiti, Ekiti State and the typed enteric bacteria isolates (Escherichia coli ATCC 25922, *Klebsiella pneumonia* ATCC 13883, *Proteus vulgaris* ATCC 29905, *Pseudomonas aeruginosa* ATCC 10145, *Salmonella typhi* ATCC 14028) was obtained from Federal Institute of Research, Oshodi (FIRO). The bacteria isolates were kept on already prepared nutrient agar slants and transported immediately to the microbiology laboratory of the Federal University of Technology, Akure, Ondo State for further analysis. These organisms were confirmed by biochemical tests.

### 2.6 Determination of the Antibacterial Activity of *A. muricata* Bark Extracts

The sensitivity of each of the extracts was determined using agar well diffusion. The ethanol, aqueous and methanol extracts was reconstituted with 30% Dimethyl sulfoxide (DMSO) to obtain varying concentration [13].

The bacterial isolates were grown in nutrient broth and adjusted to 0.5 McFarland's standard solution. Small volume of bacterial suspensions were swabbed on each already prepared

Mueller-Hilton agar plate by means of sterile cotton swab making sure they were evenly spread on the surface of the agar plate. This procedure was repeated by streaking two times, rotating the plate approximately 60° each time to ensure an even distribution of the inoculums. The agar wells were bored using a sterile corkborer with 6mm diameter on the solidified agar medium. 200 mg/ml of the leaf and bark extract was prepared using a reconstituting solvent of 30% Dimethyl sulfoxide (DMSO). 0.2 ml of each of the extracts was carefully added into the wells of labeled plates and holes. The plates were allowed to stand on the work bench for 1 hr to allow proper inflow of the extract into the medium before incubation. Plates were incubated in an upright position at 37ºC for 24 hrs. DMSO was used as the negative control while ciprofloxacin was used as positive control. After overnight incubation, zones of inhibition formed on the surface of the plates were measured in millimeter [14].

### 2.7 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Bark Extract

The MIC and MBC of the extracts was carried out using tube dilution technique. To 1 ml of graded concentrations (200 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml) of the extracts, 10 ml of 24 hrs Mueller-Hinton broth was added and a loopful of test organisms previously diluted was introduced into the tubes. Ciprofloxacin was included as positive control and distilled water as negative control in different tubes. A tube containing only nutrient broth was seeded with test organism to serve as positive control while a tube that was not inoculated served as the negative control. All the broth cultures were incubated at 37ºC for 24 hrs. After incubation the tubes were examined for microbial growth by observing for turbidity using spectrophotometer. Growth inhibition was indicated by low turbidity while growth was indicated by high turbidity. From each of the set of test tubes used for the determination of MIC, a loopful of broth was collected from the tubes that do not show any visible growth and was inoculated on sterile Mueller-Hinton agar plates. The plates were incubated at 37ºC for 24 hrs. After incubation, the least concentration that showed no growth was recorded as the minimum bactericidal concentration (MBC) [14].

### 2.8 Antibiotic Sensitivity Pattern

Antibiotic sensitivity testing was performed using disc diffusion method. Standard antibiotic discs for gram negative bacteria were used against the bacteria isolates. These antibiotics include pefloxacin 30 µg (PEF), gentamycin 30 µg (CN), augmetin 10 µg (AU), sparfloxacin 10 µg (SP), amoxacillin 30 µg (AM), chloramphenicol 30 µg (CH), ciprofloxacin 30 µg (CPX), streptomycin 30 µg (S), septrin 30 µg (SXT) and tarivid 10 µg (OFX). The inoculum was prepared by emulsifying three to four discrete colonies of each test isolate in a sterile test tube containing peptone water and incubated for 30 minutes. The suspension was adjusted to match with 0.5 McFarland turbidity standards after which the peptone water isolate suspension was poured into a freshly prepared Mueller-Hilton agar plate and swirled gently to cover the surface of the agar. Then, the antibiotic discs was placed aseptically on the surface of the inoculated plate using a sterile forceps and pressed lightly to ensure contact with the agar surface. The plate was incubated at 37ºC for 24 hours. After incubation, clear zones of inhibition were measured in millimeter and areas without clear zones were observed. Inoculated plate without antibiotics served as control [4].

### 2.9 Statistical Analysis

Data obtained were subjected to One Way Analysis Of Variance (ANOVA) while the means were compared with Duncan's New Multiple Range Test at 95% confidence interval using Statistical Package for Social Sciences version 23.0. Differences were considered significant at $p \leq 0.05$.

## 3. RESULTS

### *3.1* Percentage Recovery of Aqueous, Ethanol and Methanol Bark Extracts of *A. muricata*

Table 1 revealed the percentage recovery of the bark extracts of *A. muricata* after extraction. The table showed that more extracts was recovered with methanol and ethanol than water with 42.92%, 35.18% and 7.16% respectively. Extraction of *A. muricata* bark gave a percentage yield of 42.92%, 35.18% and 7.16% for methanol, ethanol and aqueous bark extracts respectively.

**Table 1. Percentage recovery of bark extracts of *A. muricata***

| Solvents | Percentage (%) |
|---|---|
| Aqueous | 7.16% |
| Ethanol | 35.18% |
| Methanol | 42.92% |

### 3.2 Qualitative and Quantitative Phytochemical Screening of *A. muricata* Bark Extracts

Phytochemical screening of the aqueous, ethanol and methanolic extracts of *A. muricata* bark is presented in Table 2. The results revealed that saponin, tannin, flavonoid, terpenoid and cardiac glycosides were present in the aqueous, ethanol and methanol bark extracts of *A. muricata* while phlobatannin, alkaloid and Steroid were absent in all the plant extracts.

Table 3 showed the quantitative phytochemical (mg/100 g) composition of aqueous, ethanol and methanol crude extracts obtained from bark of *A. muricata*. The result revealed that Saponin, tannin, terpernoid, glycosides and flavonoid has the highest values (14.00±0.18, 6.42±0.05, 28.16±0.01, 34.67±0.02 and 3.72±0.01) respectively in ethanol extract. Glycosides has the highest values (7.06±0.04 and 19.35±0.01) in aqueous and methanol extracts while flavonoid has the least values (0.38±0.01, 3.72±0.01 and 1.86±0.01) in aqueous, ethanol and methanol extracts.

### *3.3* Antibacterial Activity of *A. muricata* Bark Extracts

Table 4 shows the susceptibility patterns of the bacteria isolates to aqueous, ethanol and methanol bark extracts (crude) of *A. muricata* at 200 mg/ml measured by zone of inhibition in Plate 1, Plate 2, Plate 3, Plate 4, Plate 5 and Plate 6.

The aqueous bark extract does not show any zone of inhibition on all the test organisms (clinical and typed isolates) while all the isolates were susceptible to ethanol and methanol bark extracts. The ethanol bark extracts showed inhibition diameter ranging from (8.67 mm to 24.33 mm) with the highest zone of inhibition on *S. typhi* (24.33 mm) and least zone of inhibition on *K. pneumoniae* (8.67 mm). The methanol bark extract ranges from (7.67 mm to 23.33 mm) with the highest zone of inhibition on *P. aeruginosa* (23.33 mm) and least zone of inhibition on *K. pneumoniae* (7.67 mm).

Table 5 revealed the result of susceptibility patterns of the bacteria isolates to ethanol and methanol bark extracts (purified) of *A. muricata* at 200 mg/ml. The purified extracts showed increase in the inhibitory effect of the plant against all isolates. The susceptibility patterns of the ethanol bark extracts showed inhibition diameter ranging from (9.00 mm to 25.00 mm) with the highest on *S. typhi* and least on *K. pneumoniae* while the susceptibility pattern of the methanol bark extract of the plant on the bacteria isolates ranges from (8.00 mm to 24.00 mm) with the highest on *P. aeruginosa* and least on *K. pneumoniae.*

### 3.4 Determination of Minimum Inhibitory Concentration (MIC) and Minimum bactericidal Concentration (MBC) of Bark Extracts

Table 6 shows the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) o *A. muricata* bark extracts on test organisms (clinical and typed bacteria isolates).

**Table 2. Qualitative phytochemical screening of aqueous, ethanol and methanol bark extract of *A. muricata***

| | Aqueous | Ethanol | Methanol |
|---|---|---|---|
| Saponin | + | + | + |
| Tannin | + | + | + |
| Phlobatannin | - | - | - |
| Flavonoid | + | + | + |
| Steroid | - | - | - |
| Terpenoid | + | + | + |
| Alkaloid | - | - | - |
| **Cardiac Glycoside** | | | |
| Keller kiliani test | + | + | + |
| Salkwoski test | + | + | + |
| Lieberman test | - | - | - |

*Key: + = present, - = Negative*

**Table 3. Quantitative phytochemical composition of *Annona muricata* bark crude extract**

| Phytochemical | Aqueous (mg/100 g) | Ethanol (mg/100 g) | Methanol (mg/100 g) |
|---|---|---|---|
| Saponin | 4.09±0.27$^{a}$ | 14.00±0.18$^{c}$ | 8.55±0.18$^{b}$ |
| Tannin | 1.76±0.00$^{a}$ | 6.42±0.05$^{c}$ | 3.82±0.01$^{b}$ |
| Terpenoid | 5.12±0.01$^{a}$ | 28.16±0.01$^{c}$ | 15.36±0.01$^{b}$ |
| Glycosides | 7.06±0.04$^{a}$ | 34.67±0.02$^{c}$ | 19.35±0.01$^{b}$ |
| Flavonoid | 0.38±0.01$^{a}$ | 3.72±0.01$^{c}$ | 1.86±0.01$^{b}$ |
| Alkaloid | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ |
| Steroid | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ |
| Phlobatannin | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ |

*Data are represented as mean ± standard error (n=3). Superscript of the same alphabet in the same row are not significantly different (P<0.05) while different alphabet denotes significant difference*

**Table 4. Antibacterial activity of bark extracts (Crude) at 200 mg/ml**

| Isolates | Aqueous | Ethanol | Methanol | Control |
|---|---|---|---|---|
| *Pseudomonas aeruginosa* | 0.00±0.00$^{a}$ | 19.00±0.33$^{b}$ | 23.33±0.33$^{c}$ | 31.00±0.33$^{d}$ |
| *Salmonella typhi* | 0.00±0.00$^{a}$ | 24.33±0.33$^{c}$ | 19.33±0.33$^{b}$ | 34.67±0.88$^{d}$ |
| *Shigella sp* | 0.00±0.00$^{a}$ | 20.33±0.33$^{b}$ | 22.67±0.33$^{b}$ | 42.33±1.46$^{c}$ |
| *Escherichia coli* | 0.00±0.00$^{a}$ | 15.00±0.58$^{b}$ | 16.33±0.33$^{b}$ | 24.00±0.58$^{c}$ |
| *klebsiella pneumoniae* | 0.00±0.00$^{a}$ | 8.67±0.33$^{b}$ | 7.67±0.33$^{b}$ | 24.00±0.58$^{c}$ |
| *Proteus vulgaris* | 0.00±0.00$^{a}$ | 14.00±0.58$^{b}$ | 15.00±0.58$^{b}$ | 28.33±0.58$^{c}$ |
| *P. vulgaris* ATCC 29905 | 0.00±0.00$^{a}$ | 14.33±0.33$^{b}$ | 13.00±0.58$^{b}$ | 34.33±0.88$^{c}$ |
| *P. aeruginosa* ATCC 10145 | 0.00±0.00$^{a}$ | 14.67±0.33$^{b}$ | 15.67±0.33$^{b}$ | 44.00±0.58$^{c}$ |
| *S. typhi* ATCC | 0.00±0.00$^{a}$ | 13.00±0.33$^{b}$ | 14.00±0.58$^{b}$ | 34.00±3.66$^{c}$ |
| *E. coli* ATCC 25922 | 0.00±0.00$^{a}$ | 19.00±0.58$^{c}$ | 15.00±0.58$^{c}$ | 34.00±0.58$^{d}$ |
| *K. pneumoniae* ATCC | 0.00±0.00$^{a}$ | 15.33±0.33$^{b}$ | 14.00±0.58$^{b}$ | 28.00±0.58$^{c}$ |

*Data are represented as mean ± standard error (n=3). Superscript of the same alphabet in the same row are not significantly different (P<0.05) while different alphabet denotes significant difference*

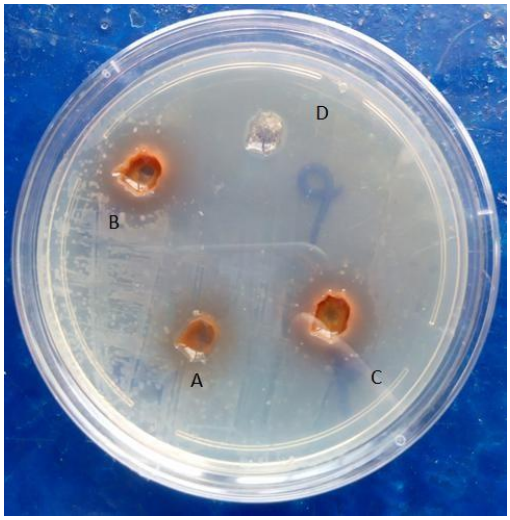


**Plate 1. Zones of inhibition indicating antibacterial activities of aqueous, ethanol and methanol extracts of *A. muricata* bark on *Pseudomonas aeruginosa***

*Key: A= Aqueous, B= Ethanol, C= Methanol, D= Control (Ciprofloxacin)*

The MIC of both ethanol and methanol bark extract of the bacteria isolates ranged from 25 to 50 mg/ml. The MIC for ethanol bark extract of the plant showed that *P. aeruginosa, E. coli, Shigella* sp, *P. aeruginosa* ATCC 10145, *E. coli* ATCC 25922 and *S. typhi* ATCC 14028 had their MIC at 25 mg/ml while *P. vulgaris, K. pneumoniae, S. typhi, P. vulgaris* ATCC 29905 and *K. pneumoniae* ATCC 13883 had their MIC at 50 mg/ml. Similarly, the methanol bark extract of the

plant showed MIC of 25 mg/ml on *K. pneumoniae,* P. *aeruginosa, E. coli, Shigella* sp, *P. aeruginosa* ATCC 10145, *E. coli* ATCC 25922 and *S. typhi* ATCC 14028 while others had their MIC at 50 mg/ml.

Also, the (MBC) of the ethanol and methanol bark extract of *A. muricata* ranged from 50-100 mg/ml. For the ethanol bark extract, *P. vulgaris*, *K. pneumoniae*, *P. aeruginosa*, *E. coli*, *S. typhi*, *P. aeruginosa* ATCC 10145 and *K. pneumoniae* ATCC 13883 had their MBC at 100 mg/ml while other isolates had their MBC at 50 mg/ml. Only *P. vulgaris* and *P. vulgaris* ATCC 29905 had their MBC at 100 mg/ml for methanol bark extract while others had their MBC observed at 50 mg/ml.

### 3.5 Antibiotic Sensitivity Pattern

Table 7 shows the sensitivity patterns of the Gram negative bacterial isolates to conventional antibiotics. All the isolates were susceptible to ciprofloxacin (CPX) and Pefloxacin (PEF) with the highest diameter zone of inhibition (29.00 mm) on *P. aeruginosa* ATCC 10145 and

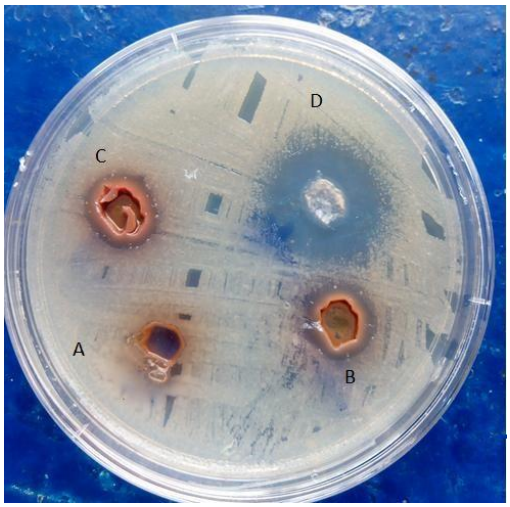


**Plate 2. Zones of inhibition indicating antibacterial activities of aqueous, ethanol and methanol extracts of *A. muricata* bark on *Escherichia coli***

*Key: A= Aqueous, B= Ethanol, C= Methanol, D= Control (Ciprofloxacin)*

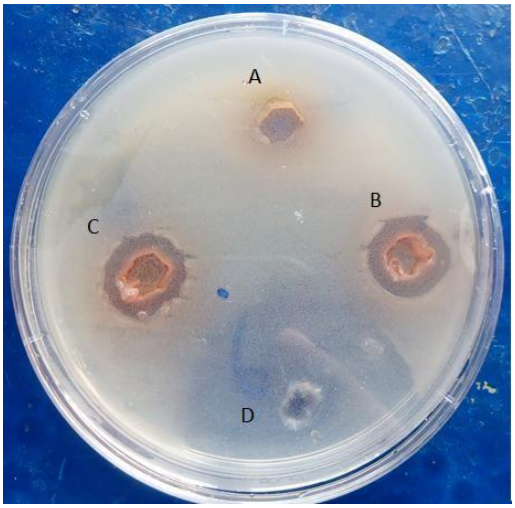


**Plate 3. Zones of inhibition indicating antibacterial activities of aqueous, ethanol and methanol extracts of *A. muricata* bark on *Salmonella typhi***

*Key: A= Aqueous, B= Ethanol, C= Methanol, D= Control (Ciprofloxacin)*

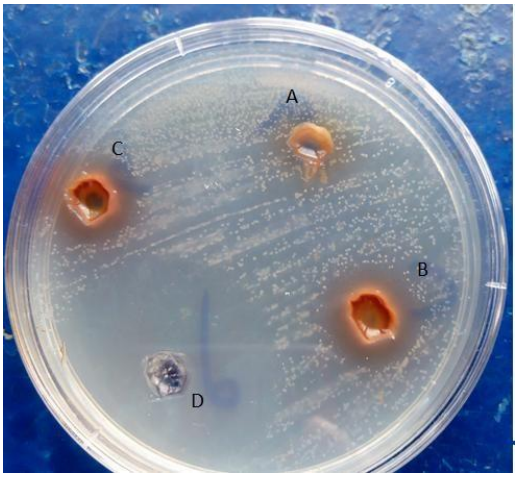


**Plate 4. Zones of inhibition indicating antibacterial activities of aqueous, ethanol and methanol extracts of *A. muricata* bark on *Shigella sp***

*Key: A= Aqueous, B= Ethanol, C= Methanol, D= Control (Ciprofloxacin)*

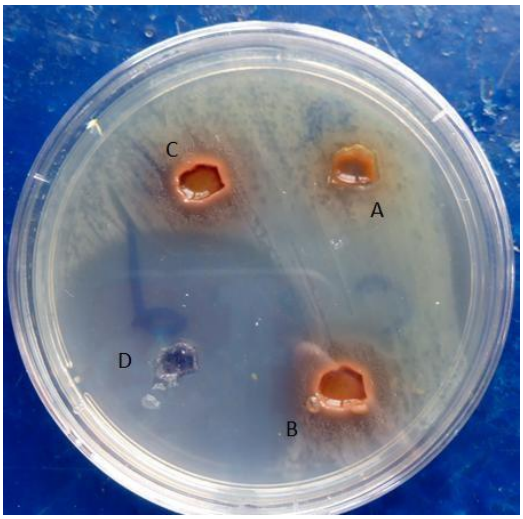


**Plate 5. Zones of inhibition indicating antibacterial activities of aqueous, ethanol and methanol extracts of *A. muricata* bark on *Proteus vulgaris***

*Key: A= Aqueous, B= Ethanol, C= Methanol, D= Control (Ciprofloxacin)*

*Shigella* Sp (25.67 mm) respectively. *E. coli*, *S. typhi*, and *P. aeruginosa* ATCC 10145 were resistant to streptomycin (S) while others were susceptible with highest diameter zone of inhibition (22.67 mm) on *Shigella* Sp. only *S. typhi* was resistant to sparfloxacin (SP) while others were susceptible with diameter zone of inhibition ranging from (13.67 mm to 25.67 mm). Highest zone of inhibition was observed on *E. coli*.

## 4. DISCUSSION

Antimicrobial compounds are amply available in medicinal plants as documented [15]. Thus, this work offers a guide to the extraction, phytochemical screening, purification and antibacterial activity of *A. muricata* bark extracts. The high percentage recovery of methanol and ethanol compared to aqueous extract could be due to methanol's and ethanol's ability to dissolve more of the active components of the plant than water [16].

Phytochemical screening of the crude extracts of *A. muricata* bark revealed the presence of some bioactive components such as saponin, tannin, flavonoid, terpenoid and cardiac glycosides. This is in agreement with the work of Vijayameena [17] who reported similar bioactive compounds in

the same plant. These compounds are known to exhibit medicinal, physiological, biological and therapeutic properties [18]. It has been reported that flavonoids are free radical scavengers that prevent oxidative cell damage [19,20]. Tannin are used as astringents, against diarrhoea as diuretics, against stomach and duodenal tumours, [20]. Terpenoids are lipophilic compounds with bacterial cell memebrane disruption potential [21]. Cardiac glycosides are important class of naturally occurring drugs whose actions helps in the treatment of congestive heart failure [22]. The presence of cardiac glycosides in this study is in agreement with the findings of Solomon-Wisdom [23] who reported the presence of cardiac glycosides in the aqueous and methanolic extracts of *A. muricata.* Abundant presence of tannin, saponin, flavonoid, tepernoids and cardiac glycosides in ethanolic bark extract of *A. muricata* compared to other solvents conformed to the report of Vimala [24] who stated that ethanolic extract of *A. muricata* has higher composition of secondary metabolite such as flavonoid, tannin and saponin. Result of this study have shown that aqueous is not a preferable solvent for the extraction of phytochemicals from *A. muricata* bark. Similarly, Salisu [25] reported that a lesser polar solvent (ethanol and methanol) extracts more phytochemicals from the stem bark of the plant.

The susceptibility patterns of clinical and typed enteric bacteria isolates to bark extracts (crude) of *A. muricata* at 200mg/ml showed variations in the zone of inhibition for each extracts. Ethanol bark extract demonstrated a higher activity on the test organisms than aqueous and methanol extracts. The poor activities of the aqueous extract against the bacteria isolates observed in this study is in agreement with the study of Busani [26] who documented that aqueous extract of plants generally exhibit little or no antimicrobial activities against micro-organisms. Clinical isolates were observed to be more susceptible to both ethanol and methanol

**Table 5. Antibacterial activity of bark extract (Purified) at 200 mg/ml**

| Isolates | Ethanol | Methanol | Control |
|---|---|---|---|
| *Pseudomonas aeruginosa* | 21.67±0.33$^{a}$ | 24.00±0.57$^{b}$ | 31.33±0.88$^{c}$ |
| *Salmonella typhi* | 25.00±0.58$^{b}$ | 21.00±0.58$^{a}$ | 35.00±1.15$^{c}$ |
| *Shigella sp* | 21.67±0.88$^{a}$ | 23.67±0.67$^{a}$ | 31.00±0.57$^{b}$ |
| *Escherichia coli* | 15.67±0.33$^{a}$ | 16.33±0.33$^{a}$ | 24.67±1.20$^{b}$ |
| *klebsiella pneumonia* | 9.00±0.58$^{a}$ | 8.00±0.58$^{a}$ | 26.58±0.88$^{b}$ |
| *Proteus vulgaris* | 17.00±0.58$^{a}$ | 17.33±0.33$^{a}$ | 28.00±0.58$^{b}$ |
| *P. vulgaris* ATCC 29905 | 15.67±0.33$^{a}$ | 14.33±0.33$^{a}$ | 34.67±1.20$^{b}$ |
| *P. aeruginosa* ATCC 10145 | 16.33±0.33$^{a}$ | 16.67±0.88$^{a}$ | 38.67±0.88$^{b}$ |
| *S. typhi* ATCC | 16.00±0.58$^{a}$ | 15.00±0.57$^{a}$ | 35.33±0.88$^{b}$ |
| *E. coli* ATCC 25922 | 21.33±1.20$^{b}$ | 16.00±1.15$^{a}$ | 35.67±1.45$^{c}$ |
| *K. pneumoniae* ATCC | 17.33±0.88$^{a}$ | 16.00±0.58$^{a}$ | 27.00±0.58$^{b}$ |

*Data are represented as mean ± standard error (n=3). Superscript of the same alphabet in the same row are not significantly different (P<0.05) while different alphabet denotes significant difference*

**Table 6. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of bark extracts (mg/ml)**

| Organisms | MIC | | MBC | |
|---|---|---|---|---|
| | Ethanol | Methanol | Ethanol | Methanol |
| *Proteus vulgaris* | 50 | 50 | 100 | 100 |
| *Klebsiella pneumoniae* | 50 | 25 | 100 | 50 |
| *Pseudomonas aeruginosa* | 25 | 25 | 100 | 50 |
| *Escherichia coli* | 25 | 25 | 100 | 50 |
| *Shigella sp* | 25 | 25 | 50 | 50 |
| *Salmonella typhi* | 50 | 50 | 100 | 50 |
| *P. vulgaris* ATCC 29905 | 50 | 50 | 100 | 100 |
| *P. aeruginosa* ATCC 10145 | 25 | 25 | 50 | 50 |
| *S. typhi* ATCC | 50 | 50 | 100 | 50 |
| *E. coli* ATCC 25922 | 25 | 25 | 50 | 50 |
| *K. pneumoniae* ATCC | 25 | 25 | 50 | 50 |

**Table 7. Antibiotics sensitivity patterns showing diameter of zone of inhibition**

| Bacteria | PEF | OFX | S | SXT | CH | SP | CPX | AM | AU | CN |
|---|---|---|---|---|---|---|---|---|---|---|
| EcC | 25.33±0.58$^{g}$ | 21.33±0.67$^{e}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 25.67±0.67$^{fi}$ | 24.00±0.58$^{fg}$ | 17.50±0.58$^{e}$ | 14.33±0.33$^{c}$ | 19.67±0.33$^{e}$ |
| KpC | 20.33±0.58$^{de}$ | 0.00±0.00$^{a}$ | 14.67±0.58$^{c}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 15.67±0.67$^{c}$ | 14.00±0.58$^{b}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 17.33±0.33$^{d}$ |
| PaC | 16.67±0.58$^{c}$ | 20.33±0.58$^{e}$ | 20.00±0.58$^{e}$ | 21.67±0.33$^{f}$ | 20.00±0.58$^{d}$ | 21.67±0.88$^{f}$ | 23.00±0.58$^{f}$ | 15.00±0.58$^{d}$ | 19.67±0.67$^{d}$ | 18.00±0.58$^{d}$ |
| StC | 20.33±0.58$^{de}$ | 20.00±0.58$^{d}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 24.67±0.33$^{fg}$ | 0.00±0.00$^{a}$ | 0.00±0.00 | 0.00±0.00$^{a}$ |
| SsC | 25.67±0.58$^{g}$ | 23.67±0.57$^{f}$ | 22.67±0.33$^{g}$ | 14.67±0.33$^{c}$ | 23.00±0.58$^{e}$ | 17.00±0.58$^{de}$ | 27.00±0.33$^{h}$ | 16.00±0.58$^{de}$ | 19.33±0.33$^{d}$ | 19.33±0.33 |
| PvC | 14.67±0.58$^{b}$ | 14.33±0.58$^{b}$ | 15.00±0.58$^{c}$ | 20.33±0.33$^{e}$ | 0.00±0.00$^{a}$ | 14.33±0.33$^{b}$ | 18.33±0.89$^{c}$ | 12.00±0.58$^{b}$ | 0.00±0.00$^{a}$ | 10.33±0.33$^{b}$ |
| KpT | 19.00±0.58$^{de}$ | 19.33±0.57$^{d}$ | 14.67±0.33$^{c}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 14.33±0.33$^{b}$ | 19.00±0.89$^{d}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 16.67±0.33$^{d}$ |
| PvT | 21.00±0.58$^{d}$ | 16.33±0.58$^{c}$ | 17.00±0.58$^{e}$ | 18.33±0.67$^{d}$ | 0.00±0.00$^{a}$ | 18.33±0.33$^{e}$ | 21.33±0.67$^{e}$ | 17.00±0.58$^{e}$ | 0.00±0.00$^{a}$ | 14.00±0.33$^{c}$ |
| PaT | 23.33±0.67$^{f}$ | 15.00±0.58$^{b}$ | 0.00±0.00$^{a}$ | 0.00±0.00$^{a}$ | 15.00±0.58$^{c}$ | 13.67±0.33$^{b}$ | 29.00±0.58$^{i}$ | 11.00±0.58$^{b}$ | 12.00±0.58$^{b}$ | 14.33±0.58$^{c}$ |
| EcT | 23.33±0.58$^{f}$ | 20.33±0.58$^{de}$ | 16.00±0.58$^{e}$ | 14.67±0.33$^{c}$ | 15.00±0.58$^{c}$ | 22.33±0.33$^{g}$ | 27.00±0.58$^{h}$ | 17.00±0.58$^{e}$ | 13.67±0.33 | 17.33±0.88$^{d}$ |
| StT | 15.00±0.58$^{b}$ | 15.00±0.58$^{b}$ | 13.33±0.33$^{b}$ | 11.67±0.33$^{b}$ | 13.67±0.33 | 13.67±0.33$^{b}$ | 17.00±0.58$^{c}$ | 13.33±0.33$^{c}$ | 0.00±0.00$^{a}$ | 13.67±0.33$^{c}$ |

*Data are represented as mean ± standard error (n=3). Superscript of the same alphabet in the same row are not significantly different (P<0.05) while different alphabet denotes significant difference; Legend: PEF= Pefloxacin, OFX: Tarivid, S: Streptomycin, SXT= Septrin, CH= Chloramphenicol, SP= Sparfloxacin, CPX= Ciprofloxacin, AM= Amoxacillin, AU= Augmetin, CN= Gentamycin. EcC= E. coli, KpC= K. pneumoniae, PaC= P. aeruginosa, StC= S. typhi, SsC= Shigella. sp, PvC= P. vulgaris, KpT= K; pneumoniae ATCC 13883, PvT= P. vulgaris ATCC*

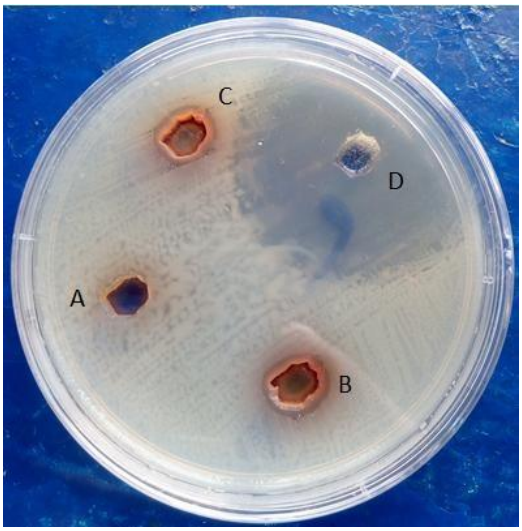


**Plate 6. Zones of inhibition indicating antibacterial activities of aqueous, ethanol and methanol extracts of *A. muricata* bark on *klebsiella pneumoniae***
*Key: A= Aqueous, B= Ethanol, C= Methanol, D= Control (Ciprofloxacin)*

extracts of the plant than the typed isolates. This may be because the clinical isolates have not been previously exposed to antibacterial agents that could have generated resistance to the extracts and antibiotics used in this study. This is in contrast with the work done by Ogundare and Oladejo [27] who reported that the clinical isolates were more resistant to *Persea americana* extracts than the typed isolates. The demonstration of antibacterial activity of bark extracts of *A. muricata* against both clinical and typed isolates used in this study provides a scientific proof of its usage in the treatment of enteric bacterial infections. The purified extracts of *A. muricata* bark showed that there was increase in the inhibitory activities of ethanol and methanol bark extracts on the test organisms. This may be as a result of the inert impure substances present in the crude extracts which could have inhibited its antibacterial activity [28]. The result of this study is in line with the study of Oseni [29] who attested that the purified *Euphorbia hirta* extracts showed significant higher antibacterial effect on tested bacterial isolates compared to the crude extracts. This result suggests that they have remarkable therapeutic action in the treatment of enteric diseases.

Findings from this study showed that the MIC of bark extracts against clinical and typed isolates was found to be (25 mg/ml) while the MBC was found to be (50 mg/ml). This result indicates that the ethanol and methanol extracts of the plant were bacteriostatic at lower concentration and bactericidal at higher concentration.

The commercial antibiotics used in this study were observed to be effective in inhibiting the test organisms. Of all the antibiotics used, ciprofloxacin (CPX) was the most effective against the test organisms (both clinical and typed isolates). The high inhibition by ciprofloxacin on clinical and typed isolate is expected because it is usually the recommended drug of choice in the treatment of enteric diseases. The high inhibition values of the antibiotics could be as a result of the purified state of the antibiotics as reported by Doughari, et al. [14] that the state of administration of an antimicrobial agent affects the effectiveness of such agent, and that antibiotics are in a refined state and plant extracts in crude state.

## 5. CONCLUSION

This study has revealed *A. muricata* bark extracts to be rich in flavonoids, tannins, saponins and cardiac glycosides as secondary metabolites which was responsible for the various antibacterial activities exhibited. Glycosides has the highest value in all the extracts. Clinical isolates were more susceptible to the plant than the typed isolates. This study confirmed that among the different solvents used, ethanol extract showed the highest antibacterial activity. The purified bark extracts of *A. muricata* had higher antibacterial activity on

the test organisms. Commercial antibiotics were effective in inhibiting the test organisms. However, the purified ethanol bark extracts can serve as a substitute to the commercially available antibiotics which can be used for the treatment of infections caused by enteric bacteria. Thus, the need for identification of the active components contained in the bark extracts and also ascertain the biosafety of the plant part.

## CONSENT AND ETHICAL APPROVAL

It is not applicable

## ACKNOWLEDGEMENT

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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