

Original Research Article

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**Phytochemical Analysis and Antibacterial Activity of
Annona muricata (Laxman phal) against ESBLs Producers
(*Escherichia coli* and *Klebsiella pneumoniae*)**

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The use of plant products as medicine has emerged from traditional days to modern times of medicine in order to improve the quality life of the patients worldwide. Nature-derived medicines are considered safer with less or no side effects. The antibacterial effect of the leaves of *Annona muricata* was evaluated on bacterial strains of ESBLs: *Escherichia coli* and *Klebsiella pneumoniae* are tested which were isolated from D.Y. Patil Hospital, Nerul, Navi Mumbai. Standard strain of *Escherichia coli* ATCC 25922 has been tested for cephalosporin (Ceftazidime- zone of inhibition: 31mm) and Imipenem (zone of inhibition: 25 mm) and is found to be sensitive. The solvents used for the extraction of plants were water (aqueous) and ethanol. The antibacterial activity was done by using Kirby-Bauer disc diffusion method. Both extracts showed antibacterial properties but the ethanolic extract was more effective as it inhibited a wide range of organisms at varying concentrations. The most susceptible Gram-negative bacteria was *Escherichia coli*. The significant antibacterial activity of plant extracts was compared with the standard antibiotic, Imipenem. The results obtained in the present study suggest that *Annona muricata* can be used in treating diseases caused by the test organisms and or can be used along with the antibiotics having synergistic activity.

Introduction

For centuries, quinine, an alkaloid obtained from the bark of various species of cinchona tree has been used in the treatment of malaria; even aspirin and morphine are plant derived drugs from willow bark and opium poppy (Sanjoy *et al.*, 2003). For two thousand years the powdered roots *Rauvolfia serpentina* has been used in treatment of mental illness in India (Ajay Kumar *et al.*, 2009).

Multidrug resistance has been a biggest threat to the medical world as bacteria are acquiring antibiotic resistance day by day. Interesting conundrums have been encountered in investigations of links between antibiotic use and the development of antibiotic resistance (Julian Davies *et al.*, 2010). Nowadays more and more bacteria are becoming resistant which were earlier sensitive to the antibiotics.

Newer antibiotics are not invented or slowdown in the process of inventing a newer molecules of antibiotics. The medicinal plants look promising as it has proved in the past like a saviour to the medical world.

Annona muricata is a member of the family of custard apple tree called Annonaceae and a species of the genus *Annona* known mostly for its edible fruits annona. *Annona muricata* produces fruits that are usually called 'sour sop' due to its slightly acidic taste when ripe. The fruit is juicy, acidic, whitish and aromatic with abundant seeds, the average weight of 1000 fresh seeds is 470g and has an average oil content of 24%. The creamy and delectable flesh of the fruit consist of 80% water, 1% protein, 18% carbohydrates and fair amount of vitamins B1, B2 and C, potassium and dietary fibre. Its flavour is described as a combination of strawberry and pineapple with sour citrus flavour contrasting with an underlying creamy flavour reminiscent of coconut or banana.

Annona muricata has been used as a folkloric herbal medicine in many regions throughout the world. It is considered to be antispasmodic and antiemetic. A decoction of *Annona muricata* leaves is used to kill bed bugs and head lice to reduce fever (Rickettsial infections or antiparasitic). This can be taken orally or adding to bathing water also has the same effect. The crushed fresh leaves are also applied on skin eruptions for faster healing. A poultice of young *Annona* leaf is applied on the skin to alleviate rheumatism and other skin infections like eczema. When applied during the healing of wounds, it results in less or no skin scars.

The decoction can also be used as a wet compress on swollen feet and other inflammations. The juice of the fruits is taken orally as an herbal remedy against arthritis, haematuria and liver ailments. Pulverizing the

Annona seed and mixing it with soap and water is used as effective spray against caterpillar. The annona leaves are placed inside the pillow or placed on top of the mattress to induce a good night sleep.

In laboratory studies, annona selectively hunts down and kills 12 different types of cancer cells, including breast, prostate, lung, colon and pancreatic cancer. In view of the usefulness of this plant, there is a need for further research on its antimicrobial properties as well as the determination of its bio-active components.

The leaves are also traditionally used to prevent and treat arthritis, asthma, bronchitis, biliary disorder, diabetes, heart diseases, hypertension, worm disease, liver disorder, malaria, rheumatism, tumour, and cancer. The leaves are also used for the treatment of several types of diseases caused by bacteria such as pneumonia, diarrhoea, urinary tract infection and other kinds of skin diseases.

The objective of this study was to evaluate the phytochemical activity and antibacterial activity of leaves of *Annona muricata* against extended beta lactamase producers (ESBL) *Escherichia coli* and *Klebsiella pneumoniae* as they are multidrug resistant. Multidrug resistance is exhibited by many bacteria and is become a big worry in the world of Medicine.

Materials and Methods

Plant material and solvent extraction

Annona muricata leaves were hand-picked from home garden in Kundapur, Udipi District, Karnataka and authenticated. The leaves were washed with distilled water, air dried and then shade dried. The dried leaves were powdered mechanically, subjected to extraction using Soxhlet apparatus with ethanol and distilled water as solvents for up

to 48 hours. 10g of leaves powder was homogenized with 100ml of solvent with a magnetic stirrer for 30 minutes. Extract was stored at 4⁰ C in airtight bottles for further studies. *Escherichia coli* is common pathogen which causes Urinary tract infections(UTI), Diarrhoea and *Klebsiella pneumoniae* causes UTI, respiratory infections and hospital acquired infections etc.

Bacterial strains

Multidrug resistant tests strains- *i.e.*, ESBLs were isolated in Microbiology laboratory of D. Y. Patil Hospital, Nerul, Navi Mumbai. Standard strains of *Escherichia coli* ATCC 25922 was taken from the stock culture. The presence of an ESBL-producing organism in an infection can result in treatment failure if one of the drugs is used (2nd Generation cephalosporins). ESBLs can be difficult to detect because they have different levels of activity against various cephalosporins. Thus, the choice of which antimicrobial agents to test is critical. For example, one enzyme may actively hydrolyze ceftazidime, resulting in ceftazidime minimum inhibitory concentrations (MICs) of 256 µg/ml, but have poor activity on cefotaxime, producing MICs of only 4 µg/ml. If an ESBL is detected, all penicillins, cephalosporins, and aztreonam should be reported as resistant, even if in vitro test results indicate susceptibility as per CLSI (Clinical and Laboratory Standards Institute) guidelines.

Antibacterial testing on ESBLs

The Whatman no.1 filter paper discs were prepared from 50µl and 100 µl of plant extracts and antibiotic sensitivity testing was done by Kirby-Bauer disc diffusion technique by inoculating the bacterial strains (turbidity of the broth was adjusted to 0.5 McFarland standard) on Mueller–Hinton agar by lawn culture and placing the discs with plant

extracts and standard antibiotics in triplicate. The plates were allowed to settle for one hour in the room temperature first, then incubated at 37⁰C for 16-24 hours. Zone of inhibition is measured in millimetres (mm) as compared against standard antibiotics.

Results and Discussion

Preliminary phytochemical analysis revealed the presence of secondary metabolites like tannins, steroids, cardiac glycosides, etc. were present in the leaves. Table 1 shows the results of the phytochemicals present in *Annona muricata* leaves. However, the present study of in- vitro antimicrobial evaluation of the leaves of *Annona muricata* forms a primary platform for further phytochemical and pharmacological studies.

The antibacterial activity of the leaves of *Annona muricata* extracts was tested in-vitro by Kirby-Bauer Disk diffusion technique against two bacterial species (ESBLs) and one standard strain of antibiotic sensitive bacteria. Table 2 summarizes the microbial growth inhibition of both ethanol and aqueous extracts.

Ethanol extract of *Annona muricata*(leaves) exhibited antibacterial activity towards all the tested bacteria with high antibacterial activity. The ethanol extracts of the investigated plants showed maximum antibacterial activity than aqueous extract for both *E. coli* and *K.pneumoniae*.

It was found that the ethanolic extract of the leaves was effective against *Escherichia coli* and *Klebsiella pneumoniae* (both are ESBL producers). The Comparative antibacterial activity between ethanolic extract of *Annona muricata* and standard antibiotic Imipenem was studied. The ethanolic extract showed significant antibacterial efficacy as compared to the standard antibiotic.

Zone of inhibition with standard strain *E. coli* ATCC 25922 showed 25mm with aqueous extract of *A. muricata* and with ethanol extract of *A. muricata* showed 27mm which is at par with standard antibiotic disc Imipenem.

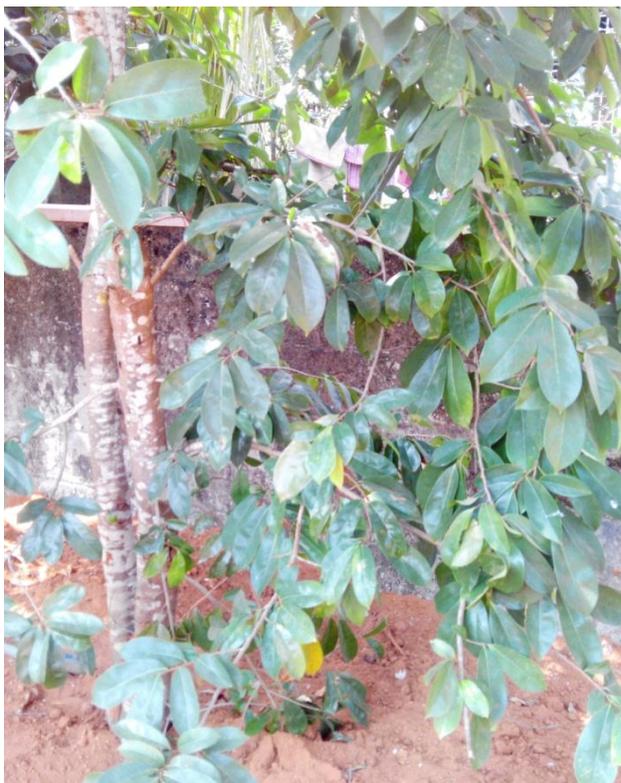
Table.1 Phytochemical analysis of the leaves of *Annona muricata*

Tests	Aqueous extract	Methanol Extract	Inference
Reducing sugars, Fehling's test	+	+	Carbohydrates Present
Starch, Iodine test	-	-	Polysaccharides Absent
Test for Steroids	+	+	Steroids Present
Keller-Killani test	+	+	Cardiac Glycosides Present
Dragendorff's test	-	-	Alkaloids Absent
Saponins Absent	-	-	Test for Saponin
Borntrager's test	-	-	Anthraquinone Glycoside Absent
Ferric chloride test	+	+	Tannins Present
Test for Phenolics	-	-	Phenols Absent
Test for Flavonoids	-	-	Flavonoids Absent

Table.2 Antibiotic susceptibility of the ethanolic (leaves) and aqueous extract (leaves) of *Annona muricata*

Test Organisms	Concentration of ethanolic extract 100 (mg/ml) Zone of inhibition measured in mm	Concentration of ethanolic extract 50 (mg/ml) Zone of inhibition measured in mm	Positive control Imipenem Zone of inhibition measured in mm	Negative control (Ethanol) 50 µl Zone of inhibition measured in mm
<i>Escherichia coli</i>	16.5 ± 0.5	14.0 ± 0.5	21.0 ± 0.5	0.00
<i>Klebsiella pneumoniae</i>	14.0 ± 0.5	13 ± 0.5	23.0 ± 0.5	0.00
Test Organisms	Concentration of aqueous extract 100 (mg/ml) Zone of inhibition measured in mm	Concentration of aqueous extract 50 (mg/ml) Zone of inhibition measured in mm	Positive control for Imipenem Zone of inhibition measured in mm	Negative control (distilled water) 50 µl Zone of inhibition measured in mm
<i>Escherichia coli</i>	14.5 ± 0.5	13 ± 0.5	21.0 ± 0.5	0.00
<i>Klebsiella pneumoniae</i>	13.5.5 ± 0.5	13.0 ± 0.5	23.0 ± 0.5	0.00

Picture.1 and 2 Photographs of *Annona muricata* (leaves and fruit)



Phytochemical analysis revealed the presence of secondary metabolites like carbohydrates, polysaccharides, steroids, cardiac glycosides and tannins which is also reported by (Julian Davies *et al.*, 2010).

Both aqueous and ethanolic leaves extract of *Annona muricata* showed antibacterial activity. Ethanolic extract showed higher antibiotic activity which is in line with the previous workers.

Significant ($P < 0.05$) antibacterial efficacy which can compete with standard antibiotics *i.e.*, Imipenem in ESBLs (*Escherichia coli* and *Klebsiella pneumoniae*), the beneficial effects of treatment can be achieved with the treatment with the leaves of *Annona muricata* in various bacterial infectious diseases like pneumonia, diarrhoea, urinary tract infection, and even some skin disease. It will require a multi-pronged approach that includes the

development of new drugs. Using plants as the inspiration for new drugs provides an infusion of novel compounds or substances for healing disease (Iwu *et al.*, 1999). *Annona muricata* (soursop) is a potent anticancer plant of Annonaceae family. The therapeutic potentials of the n-butanolic extract of *Annona muricata* were studied on WRL-68, MDA-MB-435S and HaCaT cell lines. Since most of the chemotherapeutic drugs affect normal cells as well, WRL- 68 cells were analysed for the relative cytotoxic response in with comparison that quantified in MDA-MB-435S and HaCaT cell lines. n-Butanolic leaf extract of *A. muricata* possess significant anticancer potentials in humancancerous cells. Plant phenolics are a major group of compounds that act as primary antioxidants or free radical scavengers. Broad spectrum of antibacterial activity and phytochemical activity is exhibited by *Annona muricata*. According to Parbha Pathak *et al.*, (2010)

most susceptible bacteria was *K. pneumoniae* with *Annona muricata* leaves extract. Isolation of two new Annonaceous acetogenins, annocatacin A and annocatacin B from the seeds and the leaves which is proved to be which showed in-vitro cytotoxicity towards human hepatoma cell lines (Chang *et al.*, 2003). The plant possess the major pharmacological activities includes cytotoxic, antileishmanial, wound healing, anti-microbial activity (Geetalaxmi *et al.*, 2012). ESBLs show antibiotic resistance (<10mm) in disc diffusion technique and the results with leaves extracts show better activity than cephalosporins.

In conclusion, *Annona muricata* extract possesses a broad spectrum of activity against ESBL producers (*Escherichia coli* and *Klebsiella pneumoniae*) which responsible for the most common bacterial diseases. Ethanolic extracts showed better antibacterial activity when compared with aqueous extracts. Phytochemicals present in the leaves support the antibacterial activity. These promissory extracts open the possibility of finding new clinically effective antibacterial compounds. Leaves extracts have good antibacterial activity which can supplement moderately sensitive antibiotics. So combined activity can prove to be beneficial in treating the patients suffering from ESBL producing bacteria. Leaves extract of *Annona muricata* has lot to promise in further years to come by biomedical research.

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