ANALYSING ANTI MICROBIAL ACTIVITY OF *ANONNA MURICATA* SEED EXRACT AGAINST BACILLUS SUBSTILIS AND KLEBSIELLA PNEUMONIAE ISOLATE

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ABSTRACT

Background: The development of antibiotics was a wonderful medical act during a period when many infectious diseases were destroying humanity. Because of the several vital functions that antibiotics play in the fight against diverse infections that pose a threat to human health, researchers from a variety of medical specialties have continued to focus on these drugs ever since. The emergence of bacteria that are resistant to practically all antibiotics is becoming a major worry and a global menace. Furthermore, the overuse and misuse of antibiotics, together with their serious adverse effects, are unmistakable indicators that new therapeutic techniques are desperately needed. **Aim:** To analyse anti-bacterial activity of ***Annona muricata*** seed extract with ***Bacillus substilis*** and ***Klebsiella pneumonia*** isolates. **Methodology**: The fruits of ***Annona muricata*** was collected from Coimbatore, Tamil Nadu and were identified by Department of Botany, Tamil Nadu Agriculture University Campus, Coimbatore. The powdered ***Annona muricata*** seeds were subjected to Extractive value to determine the best suitable solvent for extraction, which were determined based on the polarity of the solvents. Based on the extractive value, ethanol was selected and used in the Hot Continuous Percolation using hot Continuous percolation, and the ethanolic extract of *Annona muricata* seeds was prepared. The extract was subjected to the Agar well diffusion technique for the evaluation of the Anti-bacterial activity were performed. **Result:** The antimicrobial activities of ethanolic extracts of ***Annona muricata*** seeds have appeared to be broad-spectrum as their activities were independent of gram reaction. The crude extracts were reconstituted with 5% dimethylsulphoxide (DMSO) to prepare various concentrations of 400, 200, 100 and 50 mg/ml. The ethanolic extract of was found to be generally more effective than the control (Ciprofloxacin) against the ***Bacillus substilis*** and ***Klebsiella pneumonia***. **Conclusion:** The Anti-bacterial activity of the ***Annona muricata*** seed extract was identified by using Agar well diffusion technique.

KEYWORDS: *Annona muricata, Bacillus substilis* and *Klebsiella pneumonia*

INTRODUCTION:

The development of antibiotics was a wonderful medical act during a period when many infectious diseases were destroying humanity. Because of the several vital functions that antibiotics play in the fight against diverse infections that pose a threat to human health, researchers from a variety of medical specialties have continued to focus on these drugs ever since. The emergence of bacteria that are resistant to practically all antibiotics is becoming a major worry and a global menace. Furthermore, the overuse and misuse of antibiotics, together with their serious adverse effects, are unmistakable indicators that new therapeutic techniques are desperately needed [1]. Annona muricata is widely known as soursop due to their sour and sweet taste of its fruit. It is also known as prickly custard apple due to its taste. The seed was processed manually by depupling [2].

Bacillus subtilis is the type species of the genus ***Bacillus*** which is commonly found in diverse environments ranging from soil to the gastrointestinal tract of cattle and humans. It is a Gram-positive, rod-shaped, spore-forming, and facultative anaerobe that is the most commonly isolated ***Bacillus*** species from environmental samples [7]. ***B. subtilis*** has been extensively studied as a model for cell differentiation and engineering in biotechnology. It is also known as hay ***Bacillus*** or grass ***Bacillus*** as it is widespread in different types of grasses and hay sources ***B.subtilis*** is the most studied Gram-positive bacterium as it is studied as a model organism for studies regarding bacterial chromosome replication and transformation [3].

Klebsiella is a rod-shaped, non-motile, gram-negative bacterium. The bacterium is deadly because it possesses a capsule and is resistant to many antibiotics, disinfectants, and the environment. It contains endotoxin, capsular and somatic antigens, and a complex antigenic structure; certain strains are also capable of producing exotoxin. In lambs, these microbes can cause sepsis, conjunctivitis, meningitis, pneumonia, acute intestine infections, and urogenital infections. Illnesses with ***Klebsiella*** can also arise as a secondary infection on top of viral illnesses, which can also result in a higher death [4].

MATERIALS AND METHODS:

Collection and authentication:

The fruits of ***Annona muricata*** L was collected from Coimbatore, Tamil Nadu and were identified by Department of Botany, Tamil Nadu Agriculture University Campus, Coimbatore. The seeds isolated from fruits and shade dried at room temperature (30^0C-40^0C) [17].

Extractive value:

Extractive values are used for evaluation of crude drugs when they cannot be estimated by any other method. Extractive values by different solvents are used to assess quality, purity and to detect adulteration due to exhausted and incorrectly processed drugs [19].

Procedure for extractive value:

- ***Annona muricata*** seeds were blended into coarse powder with the help of a Mixer-Grinder.
- Iodine Flasks are cleaned, dried, and neatly labelled according to sample and solvent.
- Extracts will be made by adding the 5g of powdered seeds to 100ml of solvents such as pet ether, benzene, chloroform, methanol, ethanol, water in the separate iodine flasks respectively and it kept for maceration.
- The Iodine flask will be put in the Gyratory shaker for 5-6 hours and kept aside for 15-18 Hours.
- The iodine flask containing the extractive solutions are collected and filtered using filter paper separately and the filtrate was collected in the beaker.
- Each Petri Dish is labelled and weighed individually in a weighing balance and tabulated.
- Each filtrate of extractive solutions, 25ml is taken in a petri dish and evaporated in a hotplate.
- After evaporation of filtrate, and it kept at room temperature to cool.
- When the Petri dishes reach room temperature, the Petri dish was weighed, and the extractive value is calculated.

Hot Continuous Percolation:

- ***Annona muricata*** seeds were blended and pulverized into coarse powder with the help of a mixer-grinder.
- Round Bottom Flask were cleaned, dried and neatly labelled according to sample and solvent.
- Extracts will be made by adding the 100g of powdered seeds to 500ml of solvent (7:3 ratio of ethanol and water) in the separate round bottom flasks respectively.
- Extracted using soxhlet apparatus for 6 hours hrs till the volume reduced to half.
- Extract was filtered through Whattman’s filter paper No.1 and evaporated to dryness to get constant weight.
- After evaporation of filtrate, they are kept at room temperature to cool [5].

Phytochemical evaluation:

Phytochemical screening is the process of identifying and analysing the chemical compounds present in the plant. These compounds, known as phytochemicals, are secondary

metabolites that plants produce, which often have various biological activities. Phytochemical screening aims to detect the presence or absence of these bioactive compounds and to assess their potential medicinal, nutritional, or agricultural properties [6].

Preliminary phytochemical screening:

Test for Flavonoids:

- **Alkali test**:
  Treat test solution with increasing amount of sodium hydroxide and yellow color which
  decolorize after addition of acid. It indicates the presence of flavonoids.
- **Sulfuric acid test**:
  Add 3ml of sulfuric acid in sample and it observed the formation of red color. It indicates the
  presence of flavonoids.

Test for Phenolic Compounds:

- **Ferric chloride test:**
  Dissolve the sample in water plus methanol and add drops of a dilute solution of ferric
  chloride. If the sample turns to red, green, purple (or) blue coloration then its indicates the
  presence of alcohol.

Test for alkaloids:

- **Mayer's test**:
  Few drops of Mayer's reagent were added to 1 ml of extract. A yellowish or white precipitate
  was formed, indicating the presence of alkaloids.
- **Dragendorff''s test**:
  By adding 1ml of Dragendorff's reagent to 2ml of extract, and observed formation of orange
  or red precipitate. It indicates the presence of alkaloids.

Test for Terpenoids:

- **Salkowski test**:
  5 ml of Extract was mixed with 2ml of chloroform and 3 ml of concentrated sulphuric acid was carefully added to form a layer. A reddish-brown coloration of the inter face was formed to show positive results for the presence of terpenoids.

Test for Saponin:

- **Foam test**:

  1 ml of Extract was mixed with 20 ml distilled water. Shake the mixture for 15 minutes and observe the formation of stable foam.

Test for Anthroquinone:

2 ml of extract was mixed with 5 ml of 10% Ammonia and kept in water bath for 3 minutes. Observe the formation of pink or red colour [9].

Anti-bacterial activity:

Method : Agar well diffusion and broth dilution technique
Name of the Organism : ***Bacillus substilis*** and ***Klebsiella pneumoniae***
Name of the Standard : Ciprofloxacin
Sample : ***Annona muricata*** seed extract [13].

Chemicals and apparatus required:

- Nutrient Broth media
- Muller Hinton Agar media
- Distilled Water
- Petri plate
- Test tubes
- Conical flask
- Micro-pipette
- Borer
- Swab stick

Procedure:

Culture media:

Nutrient agar medium was used for the culturing and growth of all microorganisms used in the study. Nutrient broth was used for shaking incubation and standardization of these microorganisms [7].

Preparation of media:

The required quantities of Muller Hinton agar media was prepared and poured into conical flasks. The media flask was plugged with cotton wool and sterilized in an autoclave at 15 psi for 15 minutes at 121^0C. After sterilization, the media was poured aseptically into sterilized petri plates. A sterile environment was maintained during pouring to avoid contamination.

The medium was allowed to solidify in petri plates for about an hour before the petri plates were placed in an inverted position (to avoid evaporation of water from the medium within the plates) in an incubator at 37ºC for 24 hrs. After 24 hrs, uncontaminated plates were used for culturing of bacteria. The antimicrobial activity was performed using agar well diffusion method [14].

Microorganisms used:

1. ***Bacillus substilis***
2. ***Klebsiella pneumonia***

Both microbial stock cultures were inoculated on nutrient broth medium in a laminar flow hood, then incubated at 37ºC for 24 hrs. After 24 hrs, the cultures were again sub-cultured and incubated at 37ºC for 24 hrs [15].

Well diffusion susceptibility method:

Nutrient agar medium plates were seeded with 18-24-hour-old cultures of microbial inocula. Four wells (8 mm in diameter) were cut into the agar media with a sterilized cork borer and then plant extracts in 50, 100, 200 and 400 mg/ml volumes were poured into the wells. An antibiotic and DMSO were also poured into one well each as a positive and negative control, respectively [22]. The plates were left on the bench at ambient temperature for 1 h to allow for the diffusion of the extracts, after which they were incubated in the upright position. Inoculated plates were then incubated at 37ºC for 24 hrs and zones of inhibition were measured in mm. Three replicates were prepared for each microorganism [8].

Result:

The seed powder of ***Annona muricata*** was applied for extraction using standard procedure. The extractive value was estimated and tabulated [24].

$$\% \textbf{ Extractive value} = \frac{\text{Weight of extract obtained}}{\text{weight of drug}} X\ 100$$

Table 1: Extractive values of *Annona muricata* seeds

S. No	Solvent Used	Extractive Value
1.	Petroleum Ether	0.6% w/w
2.	Chloroform	0.4% w/w
3.	Methanol	2.2% w/w
4.	Ethanol	2.8% w/w
5.	Chloroform	2.6% w/w

6.	Benzene	2.5% w/w

Fig.1: Extraction with different solvent

Ethanolic extraction [25]:

Method : Hot continuous percolation

Apparatus : Soxhlet's apparatus

Solvent : Ethanol, Time : 6 hrs

$$\% \textbf{ Extractive value} = \frac{\text{Weight of extract obtained}}{\text{weight of drug}} \textbf{ x 100}$$

$$= \frac{15}{100} \textbf{ x 100 = 15\% w/w}$$

Fig.2: Soxhlet's extraction

Qualitative phytochemical screening:

Table 2: Phytochemical screening evaluation of the *Annona muricata* leaf extract

S. No.	NAME OF THE CHEMICAL TEST	EXTRACTED SAMPLE INFERENCE
		ETHANOL
1.	**Test for Flavonoids**	
	Alkali test	+
	Sulfuric acid test	+
2.	**Test for Phenolic Compounds**	
	Ferric chloride test	+
3.	**Test for alkaloids**	
	Mayer's test	+
	Dragendorff's test	+

4.	**Test for Terpenoids**	
	Salkowski test	+
5.	**Test for Saponin**	
	Foam test	+
6.	Test for Anthroquinone	+

Anti-bacterial activity:

The antimicrobial activities of extracts have appeared to be broad-spectrum as their activities were independent of gram reaction. The ethanolic extract was found to be generally more effective than the control (ciprofloxacin) against the ***Bacillus substilis*** and ***Klebsiella pneumonia.***

Table 3: Zone of Inhibition for *Bacillus substilis*

S. No	SAMPLE CONCENTRATION (mg/ml)	ZONE OF INHIBITION (mm)
1	50	3
2	100	6
3	200	10
4	400	18
5	Control	0
6	Standard	20

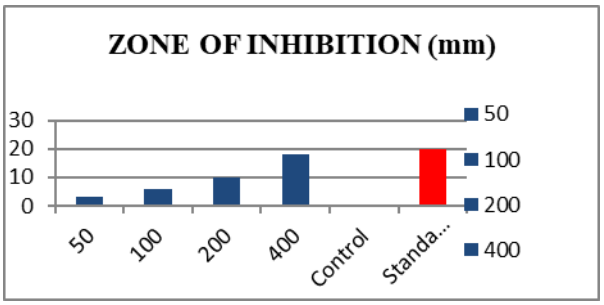

Fig.3: Zone of inhibition for *Bacillus subtilis*

Table 4: Zone of Inhibition for *Klebsiella pneumonia*

S. No	SAMPLE CONCENTRATION (mg/ml)	ZONE OF INHIBITION (mm)
1	50	3
2	100	4
3	200	9
4	400	17
5	Control	0

6	Standard	21

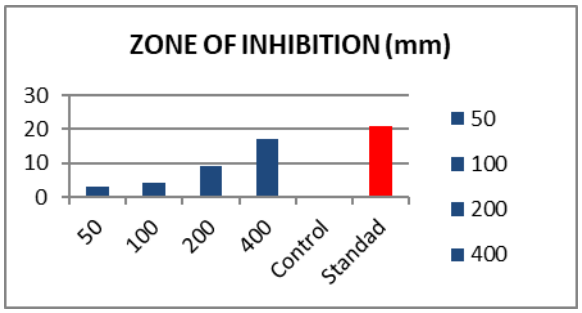

Fig. 4: Zone of inhibition for *Klebsiella pneumoniae*

Discussion:

Selection of plant material:

Annona muricata fruits were collected from Sundarapuram, Coimbatore and authenticated by a Scientist at Botanical Survey of India, TamilNadu Agricultural University Campus, Coimbatore.

Processing of plant material:

Annona muricata seeds were isolated, dried in shade and powdered.

Extractive value:

The powdered ***Annona muricata*** seeds were subjected to Extractive value to determine the best suitable solvent for extraction, which were determined based on the polarity of the solvents.

Preparation of extract:

Based on the extractive value, ethanol was selected and used in the Hot Continuous Percolation using Soxhlet apparatus, and the ethanolic extract of ***Annona muricata*** was prepared.

Preliminary phytochemical screening:

The ethanol extract was then subjected for Phytochemical screening using Qualitative Chemical tests and showed positive results for alkaloids, phenols, saponins, flavonoids and tannin were present in the extract.

Anti-bacterial activity:

The extract was subjected to the Agar well diffusion technique for the evaluation of the Anti-bacterial activity against ***Bacillus substilis and Klebsiella pneumonia***.

Conclusion:

Here, we conclude that we had done the extraction of ***Annona muricata*** seeds by using the solvent ethanol by Hot Continuous Percolation method using Soxhlet apparatus, and performed the Qualitative phytochemical test and identified the presence of Alkaloids, Phelolic compounds, Terpenoids, Flavonoids, Glycosides and Saponins in the ethanolic extract of ***Annona muricata*** seeds. The Anti-bacterial activity of the ***Annona muricata*** seed extraxt against ***Bacillus substilis*** and ***Klebsiella pneumonia*** was identified by using Agar well diffusion technique.

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