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"To study the anti-microbial activity of standardized Bhallatak Avaleha"

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Abstract:

Ayurveda is the holistic science of life. It is the oldest known health care in the world. Ayurveda branch of Atharvaveda has its own compact system of health. *Bhallatak Avaleha* is a Leha kalpana mentioned in Bhaishajya Ratnavali arsha rogadhikar adhyaya. It is a multipurpose *Ayurveda* formulation mainly used in all sorts of diseases. It is indicated in Arsha (vata, kapha type), grahni, kushta and *krimi*. It is also beneficial in various upper respiratory tract diseases like *kaas, shwaas* etc. The aim of the present study was to see the *anti-microbial* activity of Bhallatak Avaleha on different microbes. The *Bhallatak Avaleha* has good antibacterial property against the bacteria causing respiratory tract infections. It showed significant bacteriostatic activity in Streptococcus pneumonia and Streptococcus pyogenes *bacteria.*

Keywords:

Bhallatak, Ayurveda, Avaleha, Krimi, Shwaas, Kaas, Bacteria, anti-microbial activity

Introduction:

Ayurveda being the 'science of Holistic living and art of national healing' plays a vital role in the prevention and management of disease. Maharshi Charak has categorized Bhallatak as Dipaniya (an appetizer), Bhedaniya (accumulation breaking herb), mutra sangrahaniya (anti diuretic) and kusthaghna (anti dermatosis). It is also beneficial in various upper respiratory tract diseases like kaas, shwaas etc. Bhallatak Avaleha is a Leha kalpana mentioned in Bhaishajya Ratnavali arsha rogadhikar adhyaya. It is a semisolid preparation of herbal drugs prepared in decoction or extracts of different herbs by adding sweetening agents like jaggery, sugar or sugar candy. The component drugs which are used in the preparation of Avaleha are aqueous medium, substrate, *Oushada dravyas*, lipid medium, additives etc. Avaleha are intend to provide better drug absorption through the oral cavity along with absorption through villi. The aim of the present study was to see the anti-microbial activity of Bhallatak Avaleha on different microbes.

Aim:

To study the anti-microbial activity of Bhallatak Avaleha Batch A, Batch B, Batch C.

Objectives:

1. To study literature w.r.t Bhallatak Avaleha preparation**.**
2. To study various literatures on Microbiology.
3. To study the Antimicrobial activity of Bhallatak Avaleha Batch A.
4. To study the Antimicrobial activity of Bhallatak Avaleha Batch B.
5. To study the Antimicrobial activity of Bhallatak Avaleha Batch C.

MATERIALS & METHODS

Bhallatak Avaleha is prepared according to the reference of Bhaishajya Ratnavali.

Antimicrobial activity of Bhallatak Avaleha.

Antimicrobial susceptibility testing is used for drug discovery and epidemiology. Antimicrobial study of Bhallatak Avaleha was done by agar well diffusion method. Agar well diffusion method is used to evaluate the antimicrobial activity of microbial extracts or plants. Similarly, to the procedure used in disc diffusion method, the agar plate surface is inoculated by spreading a volume of the microbial inoculums over the entire agar surface. Then a hole made with a diameter of 6 to 8mm is punched aseptically with a sterile cork borer at a tip. Then, agar plates are incubated under suitable conditions depending upon the test microorganism. The antimicrobial agent inhibits the growth of the microbial strain.

Different method used to see anti-microbial activity of Bhallatak Avaleha are as follows.

I. Agar Well Diffusion Method
II. The Ditch Plate Method

A. Agar Well Diffusion Method

Introduction:

The antimicrobial study in the present sample substances are allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The zones of inhibition will be circular as there will be a confluent lawn of growth. The diameter of zone of inhibition can be measured in millimetre.

i. Test samples
ii. Selection of colonies
iii. Inoculum Suspension
iv. Culture the plate
v. Calculation of zone

Test Samples –

Bhallatak Avaleha

Selection of Colonies:

Antibacterial activity of *Bhallatak Avaleha* was selected colonies were

i. Staphylococcus aureus ATCC 6538
ii. Streptococcus pyogènes ATCC 19615
iii. iii. Streptococcus Pneumonia ATCC 6330

ATCC is an organization which collects, distributes, stores, and standard reference microorganisms. Staphylococcus aureus ATCC 6538, Streptococcus pyogenes ATCC 19615, Streptococcus Pneumonia ATCC 6330 are standard cultures.

Apparatus:

1. Petri Dish / Disks
2. Sterile cotton tipped swab
3. Incubator a maintaining temperature 2oC
4. Cork borer 8mm diameter
5. Calibrated ruler
6. Trypticase Soya Agar

iii. Inoculum Suspension –

Once isolated colonies are available from an organism that has been identified as potential pathogen, it is necessary to proceed as follows to perform the susceptibility test. The Antibacterial tests are done by NCCLS guidelines 2005.

There are two methods for inoculum preparation

i] Direct colony suspension and

ii] Long phase growth.

In this study the method used is direct colony suspension Method. Detailed description given as below –

Preparation of Inoculum Suspension:

One of the most important steps in the testing process is preparing the inoculum. This involves selecting appropriate colonies for testing, suspending them in broth, and standardizing the suspension. First *Staphylococcus aureus*, *Streptococcus* Pneumonia and Streptococcus *Pyogenes* colonies were selected for *inoculum* suspension. *Inoculum* suspension prepared by Direct colony suspension in which selected colonies mixed with diluting agent such as broth or saline. Maintain the bacteria on slant by standard procedure. Colonies should not be older than 18 to 24 hours. Using an inoculating loop, loopful bacteria was picked and inoculated in the nutrient broth.

Incubated inoculated the Nutrient broth at 37o C for 24 hours.

This broth was diluted in sterile saline to McFarland standards 0.5 approximately equal to105 cfu/ml. This culture was used for testing sample.

Turbidity of inoculum suspension was standardized with 0.5 MC Farland standards.

McFarland turbidity standards - when turbidity of test inoculum matches McFarland 0.5 standards, test inoculum contains approximately 1.5 x 105 CFU/ml.

Standardization of Inoculum Suspension:

The direct colony suspension method provides with accurate results. For both methods, the turbidity of the test suspension must be standardized to match that of a 0.5 McFarland standards. In microbiology, McFarland standards are used to adjust the turbidity of bacterial suspensions. If a suspension used is too heavy or too dilute, an erroneous result (either falsely resistant or falsely susceptible) for any given anti-microbial agents could occur.

Original A 0.5 McFarland standard is prepared by mixing 0.05 mL of 1.175%

barium chloride dehydrate ($BaCl_2 \cdot 2H_2O$), with 9.95 mL of 1% sulphuric acid (H_2SO_4),which causes turbidity in the solution.

The standard is compared with a suspension of bacteria in sterile saline. If bacterial suspension is turbid, it is diluted with diluent. More bacteria is added, If the suspension is not enough turbid.

Then adjust the inoculum to a turbidity equivalent to a 0.5 McFarland standard and turbidity of the suspension compared by placing the tube in front of white paper with black lines.

Vortex McFarland 0.5 standards and test inoculum, hold tubes side by side against white card with heavy black lines.

If lines look same through tubes, inoculum is satisfactory. If lines appear less sharp through inoculum tube, dilute with sterile saline or broth until turbidity comparable.

Tryptic Soya plate / Soybean Casein Digest Agar Preparation:

Tryptic Soy Agar (TSA) is recommended for the isolation and cultivation of microorganisms. Tryptic Soy Agar supports the growth of no fastidious as well as moderately fastidious microorganisms

Content: Ingredients per litre of deionized water

1. Pancreatic Digest of Casein 15.0gm

2. Peptic Digest of soybean meal 5.0gm

3. Sodium Chloride 5.0gm

4. Agar -15gm

5. Citrated Animal blood [contain EDTA]

Tryptic Soy Agar contains digests of casein and soybean meal. The casein and soy peptones renders the medium nutritious. It supplies organic nitrogen, particularly amino acids and longer-chained peptides. Sodium chloride is added to maintain the osmotic equilibrium and agar is the solidifying agent.

Procedure:

1. Weighted 40gm of Tryptic soy broth (TSB) powder and dissolved in 1000 ml distilled water.
2. The bottle was placed on magnetic stirrer to mix.
3. Autoclaved liquid was kept on water bath at 50°C to prevent solidification.
4. Citrated animal blood [sheep, horse, cattle] was taken and 20ml transferred in 100ml melted agar base [5% blood agar] with help of pipette, mixed it properly.
5. 20ml the mixture was poured onto the empty sterile petri plates and closed it by lid.
6. Then removed bubbles from agar plates by using flame.
7. Plates were kept about 1 hour for solidification.
8. Plates incubated at 37oC for 24 hours
9. Then plates were kept in refrigerator at 4oC for further use.
10. Tryptic soya agar with blood used for Strep. Aureus and Strep.Pneumonia. Without blood Tryptic Soy agar was used for Strep. Pyogenes.

iv. Inoculating plate:

1. Trypticase soy agar with blood was selected for Strep. Aureus and Strep. Pneumonia - The Container of disks were removed from the freezer. The disks were allowed to equilibrate to room temperature for 1 to 2 hours to minimize condensation and reduce the possibility of moisture affecting the concentration of antimicrobial agents.
2. Allow Trypticase soy agar plate to room temperature so that any excess moisture will be absorbed into the medium.
3. Three plates were selected for Strep. Aureus, strep. Pneumonia, Strep. Pyogenes and labelled them by marker.
4. Then fresh, sterile cotton tipped swab was dipped into the suspension of test bacteria - 24 hours old broth culture diluted to 107CFU/ml were individually spread over face of Trypticase soy agar of each plate. Remove to make sure it was well mixed.
5. The excess liquid was removed from the swab by pressing it against side of tube.
6. Inoculating the plate starting at the top of the soya agar plate inoculate the surface with the swab.
7. The plates were covered by streaking back and broth from edge to edge. The plates were rotated approximately 60o and repeat the swabbing procedure for three times. This was ensured that the inoculum is evenly distributed.

v. Addition of sample – Bhallatak Avaleha:

1. Using cork borer a well of 8mm diameter was punched in the medium of each plate.
2. Test material Bhallatak Avaleha approximately 100mg quantity was then applied to well of each plate.
3. The plates incubated at 28°C for 72 hours. After72hour's zone of inhibition calculated was by calibrated ruler.
4. Plates were held a few inches above a black non-reflecting surface and Measured to the nearest millimetre with a ruler.

II. The Ditch Plate Method:

An agar is poured in a petri plate, allow to solidify, and ditch cut out is made of the agar. A solution of the antimicrobial substance or a mixture of this with agar is carefully run into the ditch so as to about threequarters fill it. A loopful of each test organism is then streaked outwards from the ditch on the agar surface. Organism resistant to the antimicrobial grows right up to the ditch whereas susceptible organism shows zone of inhibition adjacent to the ditch. The width of the inhibition zone gives an indication of relative activity of the antimicrobial substance against the various test organisms.

Test procedure:

A ditch of 1cm x 9cm is cut from Muller Hinton agar / blood agar. It is then filled with 4ml of molten Muller Hinton agar mixed with sample (90% of sample with 10% medium).

The bacterial culture was streaked across the ditch.

The plates were incubated at 37C for 3 days i. e 72 hours. Zone of clearance on

and near the ditch was measured. Zone of inhibition were measured by calibrated ruler.

Observation:

Antimicrobial Study

I. Agar Well Diffusion Method:

Table Showing results by Agar Well Diffusion Method

Sample Identification	**Zone of Inhibition in mm (average)/ Growth on Product**		
	Staph. aureus	**Streptococcus pneumonia**	**Streptococcus pyogenes**
Bhallatak Avaleha –A	No zone	No zone	No zone
Bhallatak Avaleha –B	No zone	No zone	No zone
Bhallatak Avaleha –C	No zone	No zone	No zone

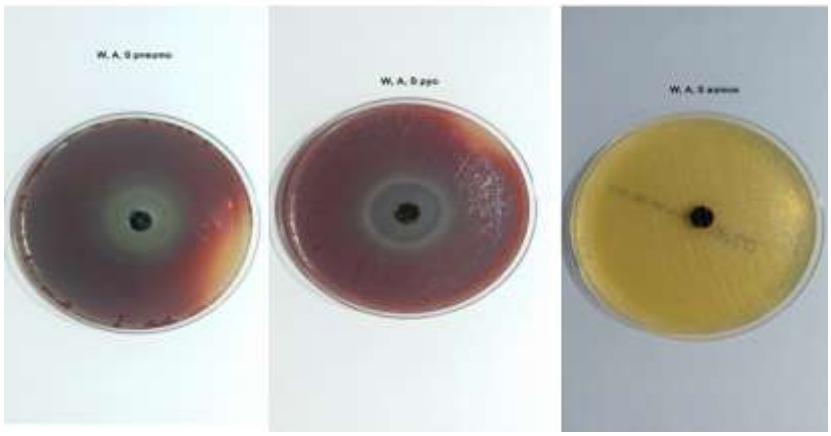
No zone was visualised by this method. So, tried doing it by another advanced method

Antimicrobial study
The Ditch Plate Method:

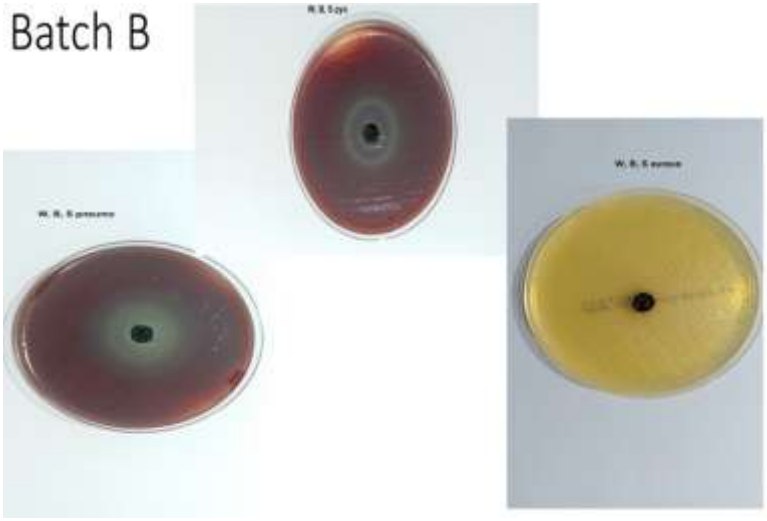
Sample Identification	Zone of Inhibition in mm (average)/ Growth on Product		
	Staph. aureus	Streptococcus pneumonia	Streptococcus pyogenes
Bhallatak Avaleha –A	No zone; Growth on product	No zone; Growth on product	No zone; Growth on product
Bhallatak Avaleha –B	No zone; Growth on product	No zone; No Growth on product	No zone; No Growth on product
Bhallatak Avaleha –C	No zone; Growth on product	No zone; No Growth on product	No zone; No Growth on product

•Formulation showed Bacteriostatic Antibacterial substance in it.
•It showed significant bacteriostatic activity in Streptococcus pneumonia and Streptococcus pyogenes bacteria.

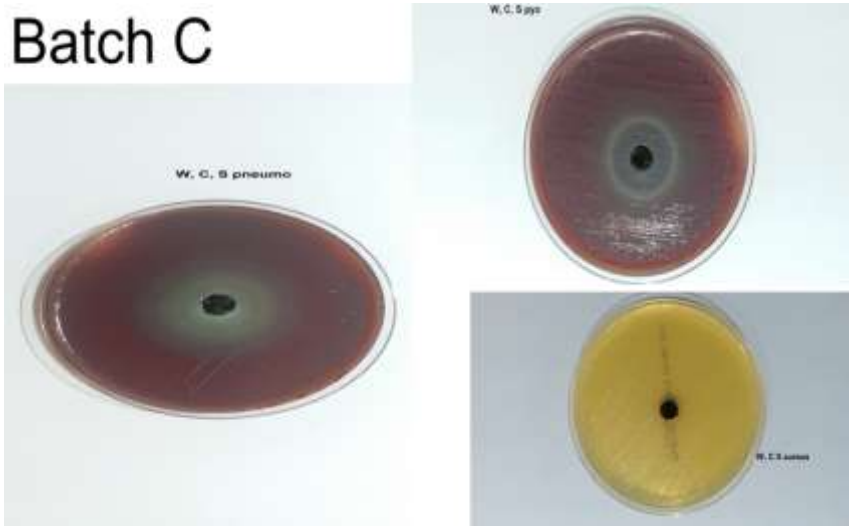
Anti microbial study Ditch plate method Batch A



Batch B



Batch C



Discussion:

The microbial limit test was done to check for any contamination present in Bhallatak Avaleha. The Results shows product is free from fungal and microbial infestations.

The Antimicrobial test was done in microbial laboratory of an authorized Institute. The test for antibacterial effect on bacteria Staphylococcus aureus, Streptococcus pyogenes and streptococcus pneumonia were done by Agar well diffusion method and the Ditch plate method.

In the Agar well diffusion method mentioned under NCCLS guidelines 2005 there was no zone seen by any of these three bacteria. So, tried doing it by another advanced method.

• In Ditch plate method, mentioned under NCCLS guidelines 2005, Batch B and Batch C of Bhallatak Avaleha showed growth on product and also showed presence of Bacteriostatic Antibacterial substance in it. The Bhallatak Avaleha has good antibacterial property against the bacteria causing respiratory tract infections. It showed significant bacteriostatic activity in Streptococcus pneumonia and Streptococcus pyogenes bacteria.

Conclusion:

Bhallatak Avaleha is a Leha kalpana mentioned in Bhaishajya Ratnavali indicated for oral use in Arsha, grahni, kushta and various upper respiratory tract diseases like kaas, shwaas etc. It is a multipurpose Ayurveda formulation mainly used in all sorts of diseases. So, in this study, the anti-microbial activity was carried out. The conclusions are drawn on the basis of observation during present study.

In standardization of Bhallatak Avaleha, Batch C showed significant results in Physico-chemical and anti-microbial testing.

Anti-microbial study by ditch plate method as per NCCLS guidelines was done, Batch B and Batch C showed Bacteriostatic Antibacterial substance in it. It showed significant bacteriostatic activity against Streptococcus pneumonia and Streptococcus pyogenes bacteria causing respiratory tract infections.

Values of Physico-chemical and microbiological parameters can be taken as measures for quality control of *Bhallatak Avaleha.*

Scope for the further Study

Further studies showed be encouraged for its clinical trial on the patients with Shwas rog, kasa (Kaphaj), krumi, *Kushtha, Ghridhrasi* and *Arsha* of *vata, kaphaj* type.

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