Research Article

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AN *IN-VITRO* STUDY ON ANTI-MICROBIAL ACTION OF APARAJITHA DHŪPA

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ABSTRACT

Air contains millions of pathogenic and nonpathogenic microorganisms. Pathogenic microorganism is responsible for various health related problems. Fumigation can be applied as a preventive and curative therapy. Traditional fumigation technique using Aparajitha Dhūpa may serve the purpose of reducing the microbial load to non-pathogenic level. The aim of the study is to investigate the Anti-microbial action of Aparajitha dhūpa against the Air born bacteria *Streptococcus pyogenes*. Aparajitha dhūpa includes eight drugs such as Pura *(Commiphora mukul)*, Dhyāma *(Cymbopogon martini)*, Vacā *(Acorus calamus)*, Sarja *(Vateria indica),* Nimba *(Azadirachta indica),* Arka *(Calotropis procera),* Agaru *(Aquilaria agallocha)* and Dhāru*(Cedrus deodara)*. A variety of laboratory methods can be used to evaluate or screen the *in-vitro* antimicrobial activity of an extract or a pure compound. Some of the most known and basic methods includes, Disc diffusion, Well diffusion, and Serial dilution methods. In this study, Serial dilution method is adopted to test the anti-microbial action of Aparajitha dhūpa. There was a marked reduction in the number of colonies in the test group compared to the control group of *Streptococcus pyogenes*. The test Petri dishes showed only 20 colonies of *Streptococcus pyogenes* in 10^{-1} dilution; whereas the control Petri dishes showed numerous colonies of *Streptococcus pyogenes* in 10^{-1} dilution in comparison to 10^{-2} dilution. The colony forming unit in 10^{-1} dilution and 10^{-2} dilution was too numerous to count. Colonies were greater than 300. Aparajitha dhūpa shows an *in-vitro* antibacterial activity in the selected organism. There is a marked inhibition in the growth of *Streptococcus pyogenes*.

Keywords: Aparajitha dhūpa*, Streptococcus pyogenes*, Serial dilution

INTRODUCTION

Air contains millions of pathogenic and nonpathogenic microorganisms. Pathogenic microorganism is responsible for various health related problems. Traditional fumigation technique using Aparajitha Dhūpa may serve the purpose of reducing the microbial load to non-pathogenic level.

Fumigation can be applied as a preventive and curative therapy. However, unlike other therapeutic procedures, the effectiveness of Ayurvedic Dhūpana (Fumigation) is not been studied with the requisite modern relevance. Fumigation is a well-known method of sterilization, wherein fumes produced from a fumigant are used to annihilate harmful micro and macro-organisms in a particular area.

Our ancient Acharya's have mentioned about some formulation for all kinds of Jvara (Fever) as said in Aṣṭāñga Hṛdayam Jvara Chikitsādhyāya. Āchārya Vagbhata mentioned about eight drugs i.e. Pura *(Commiphora mukul)*, Dhyāma *(Cymbopogon martini)*, Vacā *(Acorus calamus)*, Sarja *(Vateria indica),* Nimba *(Azadirachta indica),* Arka *(Calotropis procera),* Agaru *(Aquilaria agallocha)* and Dhāru *(Cedrus deodara)*, prepared in the form of churna (powder) called as Aparajitha dhūpa churna . All individually possess (Pura[1], Dhyāma[2], Vacā[3], Saṛja[4], Nimba[5], Arka[6], Agaru[7] and Dhāru[8]) anti-microbial activity. But the combination of the entire eight drugs is not yet studied to determine the antimicrobial activity. In texts only the names of drugs are found to be mentioned. There is no description about the dose or the method of use, which indeed is very essential. As these are not explained as expected, the methods are not found to be famously practiced or even encouraged. In the present study the potency of the drug in removing the toxins (microorganisms) from air was evaluated using advanced techniques.

MATERIALS AND METHODS

Materials Required: *Streptococcus pyogenes* - The culture used in the study was procured from Amrita School of Biotechnology and are sub cultured on Mannitol Salt Agar (MSA), by pure culture technique. Culture, maintenance and experiments were carried out in aseptic conditions. Growth Media – Mannitol Salt Agar (MSA), Muller Hinton Agar (MHA), Nutrient broth. Eight drugs i.e. Pura, Dhyāma, Vacā, Saṛja, Nimba, Arka, Agaru and Dhāru prepared in the form of churna, Petri dish, conical flask, test tubes, sterilized micro tips, Micropipette, L-shaped glass rod, absorbent & nonabsorbent cotton, parafilm, two mud bowl, tripod stand, spirit lamp, transparent connecting tubes, vacuum pump, McIntosh jar (fumigation chamber), devices for autoclaving, decontamination and Laminar Air Flow (LAF) chamber.

Drug Preparation: Study drugs were collected from authentic sources and were taxonomically identified, then washed and dried well. And they are ground to powder form and equal quantity of each drug were taken and mixed well.

PROCEDURE

A variety of laboratory methods can be used to evaluate or screen the *in-vitro* antimicrobial activity of an extract or a pure compound. Some of the most known and basic methods includes, Disc diffusion, Well diffusion, and Serial dilution methods. In this study, Serial dilution method is adopted to test the anti-microbial action of Aparajitha dhūpa.

Preparation of Mannitol Salt Agar (MSA): For the preparation of Mannitol Salt Agar (MSA), 11.1gm MSA and 100 ml distilled water is transferred into the conical flask. It is then kept inside the microwave and heated up. After that it is taken out and the mouth of the conical flask is plugged with non absorbent cotton and is sent for autoclaving. After being sterilized the conical flask containing the agar solution and the sterile petri dishes are kept inside the sterile chamber called the Laminar Air Flow. The MSA medium (20 ml) is then poured into the petri plates and is kept aside inside the Laminar Air Flow for a while with the lids of petri plates kept opened, until the medium inside the petri dish gets settled. After the medium is settled the organism (*Streptococcus pyogenes*) is sub cultured from the main source.

Sub Culture Method: Inoculation loop or Nichrome Wire Loop with a handle is cleaned with spirit and is shown to the flame, so that it turns red hot and sterile. The petri dish with the organism is opened and a gentle strike is made with the tip of the inoculation loop and the organism is collected. The organism thus collected is streaked on the petri dish having MSA medium. Then it is wrapped with parafilm and kept for incubation for 12 hours. Next day *Streptococcus* colonization was visible in the petri plate. This is considered as the main source and was kept as a reserve inside the refrigerator.

Preparation of Nutrient Broth: For the Preparation of Nutrient Broth (**Figure1),** distilled water is added to 1.35gm of nutrient broth and is mixed well and is heated by keeping it in the oven. Immediately after autoclaving it is made to cool. The cultures (main source; MSA) were stored in the Petri plate which is kept at 4^0C. With the help of sterile inoculation loop, the organism was collected and inoculated into the test tube containing nutrient broth and is allowed to incubate at 37ºC under mild shaking **(Figure 2**). The next day it was taken out from the incubator. When in need of doing the experiment with the concerned microorganism, we may prepare MHA medium and swab the nutrient broth with organism and then incubate overnight so that the petri dish is ready with organism for further experiments.

Preparation of Müeller-Hinton Agar: Of the many medium available, Müeller-Hinton Agar is considered to be the best for routine susceptibility testing of non fastidious bacteria. Müeller-Hinton agar should be prepared from a commercially available dehydrated base according to the manufacturer instructions. Immediately after autoclaving, it is allowed to cool. Freshly prepared media is poured into flat-bottomed sterile petri dishes kept on a level; horizontal surface to give a uniform depth. The agar medium should be allowed to cool to room temperature in order to avoid drying of the agar.

Preparation of Emulsion: Then by using three drops of distilled water and one small colony of *Streptococcus pyogenes* from the subculture is taken and emulsion is prepared and mixed well.

Serial Dilution Method: The number of bacteria in solution can be readily quantified by using the spread plate technique. In this technique, the sample is appropriately diluted and a small amount is transferred to an agar plate. The bacteria are then distributed evenly over the surface by a special streaking technique. After the colonies are grown, they are counted and the number of bacteria in the original sample is calculated. In the present study the test tubes are grouped into two: one is the control (C) group and another is the test (T) group. Each group contains two dilutions of organisms marked as C1, C2 and T1, T2. C1 & T1 with 10^{-1} dilution, C2 & T2 with 10^{-2} dilutions respec tively. (**Figure 3)**

Two test tubes were taken, and 9.9 ml of normal saline is added to each of them. Two of them are marked as control (C1, C2) with concentration of 10^{-1}, 10^{-2}dilution respectively. Then 0.1ml of the solution is taken in micropipette from the prepared emulsion and added to the test tube marked 10^{-1} containing 9.9ml NaCl (Sodium Chloride) and mixed well in vortex mixer. Then 0.1ml of the solution from the mixed tube is drawn and added to the second test tube marked 10^{-2} containing 9.9ml NaCl and is mixed well in vortex mixer. From each of the test tube 0.1ml of the solution is taken and spread on a plate with nutrient agar and was equally distributed in 4 petri dishes. The same process was repeated on the test group. The results are obtained after the incubation.

Petri dish Control I (a) and Test I (A) is taken and 0.1ml of 10^{-1} & 10^{-2} dilution is added respectively and it is continuously spread with the help of an L shaped glass rod. The dried surface of a Müeller-Hinton agar plate is inoculated by streaking the swab over the entire sterile agar surface. This procedure is repeated by streaking two more times, rotating the plate approximately 60^0 each time to ensure an even distribution of inoculums. After sometime the smooth movement will be restricted, this indicates the complete absorption of the solution into the MHA medium. Control I (a) and (b) are kept inside the laminar Air flow & Test I (A) and (B) are kept inside the McIntosh jar (Fumigation chamber).

Powders of all eight Dhūpana drugs were taken. 15gm is taken inside the mud bowl and with the help of spirit lamp the mud pot containing the drug is heated. Within 5 minutes the mud pot got heated up and again after 5 more minute's fumes started generating from the Dhūpana Dravya (drugs). Later the mud bowl was kept closed with the help of another mud bowl having a hole on the center to which a transparent plastic tube was connected and was joined to the McIntosh jar. A vacuum pump is connected to transparent plastic tube with the McIntosh jar and it is made sure that the McIntosh jar was completely filled with smoke (**Figure 4).** Experiment was carried out under ambient temperature and pressure. Fumigation was done continuously for half an hour and later spirit lamp was put off. All the connections were removed and all the valves of the Fumigation chamber were completely closed. Later it was given a standing period of about 3 hour. After 3 hours the Petri plates Test I (A) and Test I (B) are taken out closed with lid and sealed with parafilm and was given an overnight incubation. The results are obtained after the incubation.

RESULTS

Serial Dilution and Spread Plate Method: (Figure 5): In this method there was a marked reduction in the number of colonies in the test group compared to the control group of *Streptococcus pyogenes.* The test Petri dishes showed only 20 colonies of *Streptococcus pyogenes* in 10^{-1} dilution; whereas the control Petri dishes showed numerous colonies of *Streptococcus pyogenes* in 10^{-1} dilution in comparison to 10^{-2} dilution. The colony forming unit in 10^{-1} dilution and 10^{-2} dilution was too numerous to count. Colonies were greater than 300. (**Table1**, **Table 2 & Figure 6).** There is marked reduction in the values in test group when comparing with the control in both (10^{-1} dilution and 10^{-2} dilutions) dilutions.

DISCUSSION

From the review of literature, it was known that Air Pollution lead to a large set of adverse effects upon Air caused by human activities. In Aṣṭāṅga Hṛdaya Jvara Chikitsa, Aparajitha dhūpa is described for the management of all kind of Jvara. Since Jvara is spread through infectious agents upon Air, Acharya has found a solution to pacify the problem. The Yoga said by Acharya contains eight drugs such as Pura, Dhyama, Vaca, Sarja, Nimba, Arka, Agaru and Dharu. For the present study, this combination was selected by taking in consideration the Karma (properties) of individual drugs.

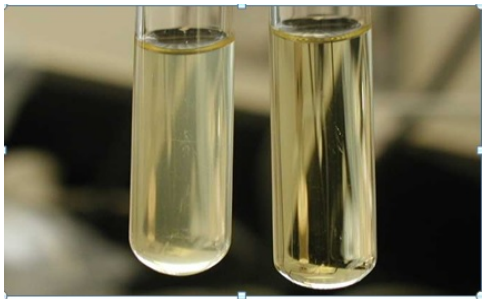

Figure 1: Inoculated Nutrient broth

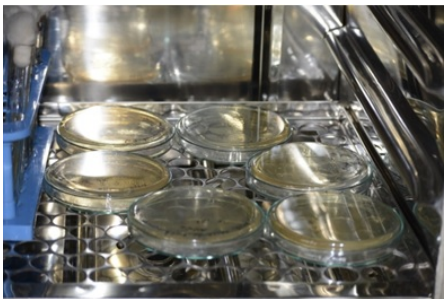

Figure 2: Nutrient Broth &Petri Plates kept Inside Incubator

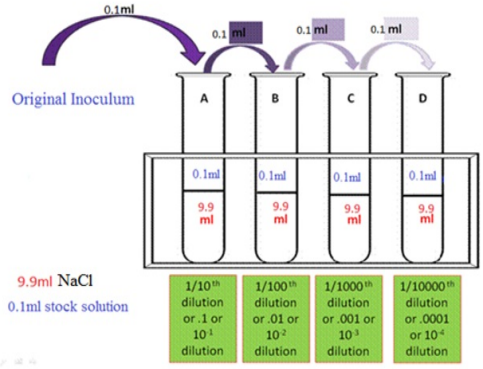

Figure 3: Serial Dilution Technique

Figure 4: Fumigation Apparatus (Mud Pot, McIntosh jar &Vacuum pump)

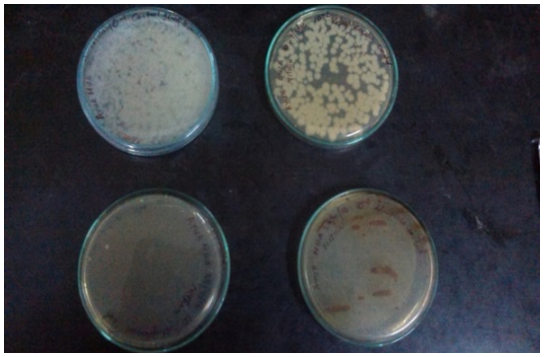

Figure 5: Representative images of Streptococcus Control and Test Plates

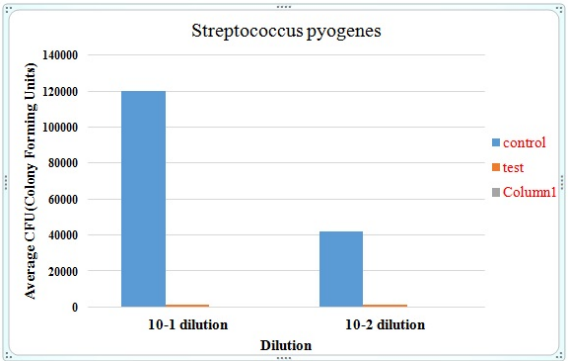

Figure 6: *Streptococcus pyogenes* results

Table 1: Result of *Streptococcus pyogenes* in control group

Dilution Factor	Spread plate method for *Streptococcus pyogenes* (Control) Number of bacterial colonies Colony Forming Units (CFUs)		Average no: CFU
	C1	C2	
10^{-1}	1.2×10^5	2.3×10^5	1.75×10^5
10^{-2}	4.2×10^4	5.3×10^4	4.75×10^4

Table 2: Result of *Streptococcus pyogenes* in Test group

Dilution Factor	Spread plate method for *Streptococcus pyogenes* (Test) Number of bacterial colonies Colony Forming Units (CFUs)		Average no: CFU
	T1	T2	
10^{-1}	2×10^1	0	10
10^{-2}	0	0	0

In the present antimicrobial study the organisms were fixed based on the literary reviews. *Streptococcus pyogenes* was the common pathogens present in the polluted Air and the effect of Dhūpa on this organism were studied. One main technique was employed in the study to find the antimicrobial sensitivity of the drug. To study the action of Aparajitha dhūpa as a whole, serial dilution method & spread plate method (TVC - Total Viable Count) was used. In this method the churna (powder) as a whole is added to the broth containing organisms. Complete reductions of colonies in test group when comparing with the control in both (10-1 dilution and 10-2 dilutions) dilutions were proved. Maximum inhibition was seen on serial dilution after spreading the emulsion and after swabbing Nutrient Broth directly. Aparajitha dhūpa shows an *in-vitro* antibacterial activity on *Streptococcus pyogenes.*

CONCLUSION

The present study is undertaken to assess the efficacy of the Aparajitha churna to remove the harmful micro-organisms from air. In the study *Streptococcus pyogenes* (as it is the common pathogens considered as indicators of air pollution) Serial dilution Method & Spread Plate (pure culture) Method were employed for the study. There was a significant reduction of growth in *Streptococcus pyogenes*. The Aparajitha dhūpa also showed inhibition of growth in *Streptococcus pyogenes.* Highly satisfactory results were seen in all the experiments. The Aparajitha dhūpa shows significant results against *Streptococcus pyogenes.* Optimization of the dose of drug, its time of exposure to microorganism and the volume of the room is essential if the test is planning to be done on open Air.

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