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PRE-CLINICAL APPRAISAL OF *PANCHASHIRISHA AGADA* FOR ANTI-MICROBIAL ACTIVITY – AN IN-VITRO TRIAL

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ABSTRACT:

Introduction: The continuous emergence of multidrug resistance microbes and fungus and negative impact of synthetic preservatives have led to an increased interest in the use of plant extract as alternative antimicrobial agents. In the present investigation antimicrobial activity of *Ayurvedic* formulation *Panchashirisha agada* has been evaluated against human pathogenic bacteria and fungus. The drug *Shirisha* (*Albizia lebeck* Benth) is the major constituent in *Panchashirisha agada*. The drug *Shirisha* (*Albizia lebeck* Benth) is having potent antimicrobial activity and is proven by many research studies. The drug is mentioned in *charaka samhita* as best *vishagna*, antidote in *Ayurveda.* **Aims and Objective:** 1) Phytochemical analysis of *Panchashirisha agada* 2) To evaluate anti-microbial activity of *Panchashirisha agada.* **Methodology:** Analysis of antibacterial activity of extract is determined by using Agar well diffusion technique for following organisms *S. aureus, S.pyogens, E.coli, P.aeroginosa*. Determination of antifungal activity is done by using cup plate method and minimum inhibition concentration for organism Candida albicans and Aspergillus. **Result:** The result from this study indicated that *S. pyogens* was the most sensitive bacteria to the *Panchashirisha agada* extract with lowest MIC concentration 50mgl/ml. The *Panchashirisha agada* extract didn't show any inhibition against *Candida albicans* and *aspergillus*. **Conclusion:** The study revealed that the *Panchashirisha agada* possess antibacterial activity in a dose dependent manner. The antimicrobial property may be due to presence of several saponins.

Key Words: *Panchashirisha agada, Albizia lebeck*, antimicrobial activity, antibacterial activity.

INTRODUCTION

Ayurveda consist of many a branch in which *Agadatantra* is one of them, which deals with toxicology. According to World Health Organization (WHO), almost 80% of people of the developing countries depend on traditional and folk medicine, herbal drugs still make an important role in developing new medicine.

In present era in spite of having a number of microbial drugs in last 3 decades there is increase in number of anti-microbial drug resistance due to over and misuse of anti-microbial. The synthetic drugs have ability to acquire resistance, Contrary to these synthetic drug, plant drug play an important role in maintenance of human health[1].

Panchashirisha agada is one of the *agada* mentioned by our *Acharya charak* in *Charak Chikitsa sthana* (23/218). He stated it as "*Vishanamprvarovadhe*"; it means it is considered to be best in treating all kind of *Vishajanya (Poisonous)* condition [2]. As of *panchashirisha agada* contains *Shirisha* (*Albizia lebbeck* (L.)Benth.). *Shirisha* is stated as best drug for *Vi shagna* property (Antidote) by *Acharya Charak* in *Charak Sutrastha* [3]. Further it is been evident by recent Researches that it has potential anti-asthmatic activity, anti-microbial activity, anti-Inflammatory activity[4].

Acharya charaka mentioned *panchashirisha agada* for management of *dushivisha*. *Acharya charaka* described as *pana (drink)* and *lepa* (for external application) to reduce toxicity. It Has both *vishaghna (anti-poisonous)* and dermatological properties like *kushthghna, kandughna, twachya, Shothghna*. Toxins can enter body in the form of *sthavaravisha, jangamavisha, dushivisha, Garavisha*, *amavisha*. Staphylococcal aureus is most common cause of cutaneous and Systematic infection. It causes many clinical conditions as a skin infections and systemic Infection.

AIMS AND OBJECTIVE

1) Phytochemical analysis of *Panchashirisha agada*

2) To evaluate anti-microbial activity of *Panchashirisha agada*

METHODOLOGY

The Materials and methods are mainly divided in following parts those are, Pharmacognostic part, Pharmaceutical part, Analytical part and Experimental part.

1. Pharmacognostic part

A) Collection and Authentication of *Shirisha*:

a) Collection of *Shirisha*: *Panchanga* of *Shirisha (Twak, Moola, Patra, Pushpa, Phala)* was collected in their respective season in the natural habitat. They are picked directly from tree undamaged, Uneaten by animals, not too small size. The obtained *panchangas* are cleaned and then dried cleaned and then dried.

b) Authentication: The *Panchangas* of plant *Shirisha* were got identified and authenticated at AYUSH approved Central Research Facility at Shri B.M.K Ayurveda Mahavidyalaya and PG center, Shahapur, Belagavi and voucher number (CRF/Auth/2532023) (CRF/Auth//254/2023) (CRF/Auth//255/2023) (CRF/Auth//256/2023)

and (CRF/Auth//257/2023) of the drugs were given in Central Research Facility**.**

B) Morphological/ organoleptic evaluation: Qualitative Evaluation based on the sensory profiles refers to observations by Colour, odor, taste and touch respectively. Here the sample was subjected for organoleptic evaluation.

2. Pharmaceutical part

It was done in Dept. of *Rasashastra* and *Bhaishajya Kalpana* of Krishna Ayurvedic Medical College and Hospital Sankeshwar.

Equipment and instruments:

Micro pulverizer, Siever, stainless steel container, gloves, mask etc.

Common precautions taken while preparing Churna:-

- Wet & infected were not used.
- Worn gloves, mask, apron & goggle
- Cleaned vessels properly after preparation.
- Weighed properly before and after *churna*.

Preparation of Panchashirisha Agada

- All the *Panchanga Churna* i.e. *Twak, Moola, Pushpa, Patra* and *Phala* are collected and taken in equal quantity in a *Kalvayantra* and pounded continuously till a uniform mixture is formed.
- Prepared powder was passed through 120 mesh size and packed in a tight container and preserved for further study.

Observation:-

Cream yellow color powder was formed

3. Analytical Study

A) Physiochemical Analysis

The prepared *panchashirisha agada churna* is subjected to Moisture content, total ash, alcohol soluble extract, water soluble extract and pH.

B) Phyto-Chemical Screening

Chemical tests were performed on both extracts (alcohol and water) obtained from using Non-polar and polar solvent. It helps to find out organic compounds like carbohydrates, proteins, Glycosides, alkaloids, steroids, tannins and phenolic Compounds, oxygenic acids, enzymes, fats And oils etc.

4. Experimental Part

4.1 Anti-Bacterial:-Cup Plate Method

Principle:-

This method depends on the diffusion of an antibiotic from a vertical cavity or cylinder, through the solidified agar layer in a petri dish. The growth of microorganism is inhibited entirely in a circular area or zone around the cavity or cylinder containing a solution of antibiotic.

Cup plate method:

The antibacterial activity of the extracts is determined by using the agar well diffusion technique. Mueller-Hinton agar plates (Himedia, Mumbai) were seeded with 0.1 ml of overnight culture of *Staphylococcus aureus, Streptococcus pyogens, Escherichia coli, Pseudomonas aeruginosa* respectively, allowed to incubate for 24hrs. Cups were made in Petri a

plate using sterile cork borer (0.85 cm) and different concentrations of the extract is added into each well. Then bacterial plates were incubated at 37*c 24 hrs.

Each test compound has got six bores which corresponds to 6 concentrations that is 100, 75, 50, 25, 10, and 5 for which zone of inhibition diameter and mean values are determined and recorded as diameter in mm.

Gentamycin/ doxycycline antibiotic was used as control:-Anti bacterial.

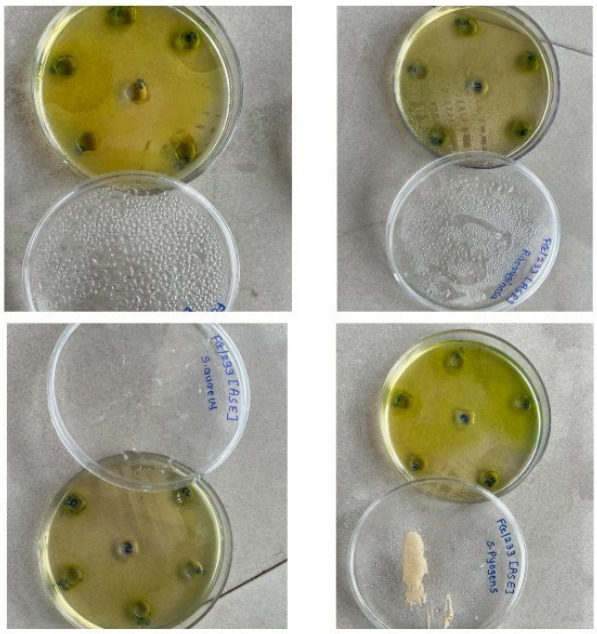

Fig 1. a) E. Coli b) P.aeroginosa c) S. pyogens d) S. aureu

Determination of minimum inhibitory concentration (MIC) of Bacteria:

The MIC of the plant extracts is determined according to the micro broth dilution technique (Murray et al., 1999). It is performed either in 96-well micro titer plates or test tubes (ependorff tubes) for determining the minimum inhibitory concentration (MIC). Standardized suspensions of the test organisms (2 gram positive and 2 gram negative for bacteria- *Pseudomonas aeruginosa*, *Escherichia coli*, Coagulase positive *staphylococci*, *Streptococcus pyogens*) is inoculated into a series of tubes or wells, including one growth (Positive control) and one sterility control (Negative control). For dilutions of the extract, different concentration of the plant extract is added to the mixture of Brain Heart Infusion (BHI) broth and the test organism. The concentrations of the plant extracts used in the study included 200µl, 150, 100, 50, 25, 12.5, 10 and incubated at 37 °C for 24 hours. After overnight incubation these tubes will be observed for turbidity. The tube showing the minimum turbidity will be noted for MIC.

Procedure

1. Dispense 800 µl BHI broth to all the tubes
2. Dispense the 100 µl fresh culture to all the tubes except NC

3. Dispense different concentrations of the drug (extract) to all the tubes except PC and NC. 200µl, 150, 100, 50, 25, 12.5, 10

4. Read the results after 24 hours for MIC: Check for presence or absence of turbidity and not down the readings.

Table no. 1 For anti-bacterial Activity:

Standard strains used:	Standard drugs used
1. *Staphylooccus aureus* ATCC 29213	1. Gentamicin (10µg)
2. *Streptococcus pyogens* ATCC 19615	2. Doxycycline (30µg)
3. *Escherichia coli* ATCC 25922	
4. *Pseudomonas aeruginosa* ATCC 27853	

4.2 Antifungal Activity

Introduction:

The antifungal activity is estimated by comparing the inhibition of growth of sensitive micro-organisms produced by known concentration of the isolated substance or extract or synthetic compound to be examined against a reference substance.

Method of Analysis:

Two general methods usually employed; one is the cup-plate method (Agar well diffusion method). The agar cup plate method depends upon diffusion of the antibiotic from a vertical agar [CUP] Cylinder through a solidified agar layer on a Petri dish. Sterile Agar is inoculated by suspension of the microbial inoculum. Then a hole with din deter of 6 to 8 mm is punched aseptically with a sterile cork borer or a tip, and then of the antimicrobial solution at desired concentration is introduced into the well. Then, agar plates are incubated under suitable conditions depending upon the test microorganism. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain entirely in a zone around the cylinder containing a solution of the substance to be tested.

Product details:

Table no.2 Product Detail for Antifungal Activity

Test	Antimicrobial Screening
Method	The Agar cup plate method
Sample	Ethanolic extract
Location	Biocyte Microbiological Testing Center, Sangli

Name of equipment

Table no. 3 Table Showing list of equipment's

Name of equipment/Instrument	
Analytical Balance	Vernier caliper
Water bath	Incubator- 20 to 25 °
Laminar Air Flow	Incubator- 30 to 35 "C
Colorimeter	Refrigerator-2 to 8 °C
Zone Reader	Cyclomixer
Sonicator	Micro-pipettes

Media and Reagents Preparation:

Antibiotic Assay Medium No. 19 (pH is 6.1 ± 0.2):

Ingredients:

Peptone	9.4
Yeast Extract	4.7 g
Beef Extract	2.4 g

Sodium Chloride	10.0 g
Dextrose	10.0 g
Agar	23.5 g
Water	1000 ml
pH after sterilization	6.1±0.1.

Prepared of medium Antibiotic Assay Medium No. 19 in 600 ml of purified water, heat boiling to dissolve the medium completely checks the pH of media. If required add sufficient 1 M sodium hydroxide or I M hydrochloric acid, distributed in 200 ml flasks as required so that after sterilization as quantity as per required for analysis., sterilized by autoclaving it at 15 lbs pressure (121°C) for 15 min.

Preparation of saline solution:

Saline Solution, 0.9% Sodium Chloride (NaCl) Dissolve 0.9 g NaCl (mw 58.44) in 70 ml deionized or distilled water in clean container. Add water to bring total solution volume to 100 ml. Make required ml aliquots in sterile 10 ml culture tubes.

Preparation of the standard solution

Standard solutions and the sample test solution simultaneously. Use the final dilutent specified and a sequence such that the middle or median has the concentration 1.0 mg/ml. The antimicrobial screening with 1 levels of the Standard requires only one level of the unknown concentration.

Note:

1. Test and standard dilutions should prepare with the same initial and final diluents.
2. Vortex the test and standard dilution after addition as well as before each use, for 1-2 min.
3. Store the solution in refrigeration at 2-80C and use on same day only.
4. Diluent used in all preparation is Dimethyl Sulphoxide (DMSO).

Preparation of standard solution [5]:

Weigh 10.00 mg of streptomycin (Equivalent to 10 mg of streptomycin and dissolve dilute with 10 ml Dimethyl Sulphoxide (DMSO) in volumetric flask i.e. 1 mg/ml solution concentration. Sonicator vortex for 1-2 min to affect the dissolution. 1mg/ml Solution A and use directly 50 µl to Inoculate.

Preparation of the sample solution

Weigh 5.00 mg /10 mg of each sample and dissolve/dilute with 5 ml Dimethyl Sulphoxide (DMSO) in volumetric flask i.e. 1mg/ml solution concentration. Vortex for 1-2 min to affect the dissolution use directly 100 µl to inoculate.

Preparation of test organism and suspension [6]:

Test organisms

Candida albicans Slant ATCC no. 10231

Aspergilus barsiliensis slant ATCC no16404

Stock culture: *Candida albicans* Slant ATCC no. 10231

Streak a loopful of suspension of *Candida albicans* on two slants of pre incubated sabourauds dextrose agar. Incubate the slants at 20-25°C for 72 hours in an incubator. **Stock culture: *Aspergilus barsiliensis* ATCC no. 16404**

Streak a loopful of *Aspergilus barsiliensis* ATCC no.16404 on, two slants of pre incubated Nutrient agar. Incubate the slants at 30-35°C for

24 hours in an incubator after incubation pick up the growth from incubated slant and inoculate in 3 ml of saline solution and vortex to prepare the uniform suspension. Adjust the O.D. of culture to approx. 60-70 % OD at 530 nm using sterile saline and calorimeter. After adjusting O.D. store the test organism in refrigeration at 2-80C Note: Approximately viable count is 10° to 10 cfu/ml against 60-70 % OD at 530 nm.

Plate preparation for analysis:

After the suspension is prepared, use each 2 ml of culture suspension of *Candida albicans, Aspergilus barsiliensis*, and is to inoculate separately in 200 ml of sterile molten and cooled medium at 40°C - 45°C Antibiotic Assay Medium No. 19. 15-20 ml of Sterilized agar medium is poured into a sterile Petri plate with the help of sterile measuring cylinder give a depth of 3 to 4 mm. Allow to cool at room temperature by placing the dishes or plates on a level surface. Keep plates in refrigerator for 15 to 20 minute for hardening. Ensure that the layers of medium are uniform in thickness. Make 4-5 agar cups on each plate using 8-10 mm SS borer. Label the plates for sample, standard and negative control samples and analysis details.

Analysis [7]:

The volume of solution added to each cylinder or cavity must be uniform and sufficient almost to fill the holes when these are used. Add 100 µl 1mg/ml Solution A to agar cup labeled as STD.

Fig. 2 *Candida albicans*

Fig.3 *Aspergillus*

Add 100 µl Img/ml = Solution B to agar cup labeled for each compound ID labeled on plate. Add 100 µl Dimethyl Sulphoxide (DMSO) to agar cup labeled as N (Negative). Leave the dishes or plates standing for 15-20 min. at 2-8°C or as appropriate, as a period of pre- incubation diffusion to minimize the effects of variation in time between the applications of the different solutions. Incubate them for about 24-48 hours at the temperature 30-35°C for bacteria and 20-25°C for yeast and mould. After completion of incubation accurately measure the diameters or

areas of the circular inhibition zones and record the results.

Table no. 4 For anti-fungal activity:

Standard strains used:	**Standard drugs used**
1. *Candida albicans* Slant ATCC no. 10231	1. Fluconazole
2. *Aspergilus barsiliensis* slant ATCC no16404	

RESULTS

1. Analytic results

1. Analytical results
2. Experimental results

Results described under following headings

Table no. 5 organoleptic characters of *Shirisha*

a) Organoleptic character of *Panchashirisha agada*

SL.NO	**TESTS**	**RESULTS**
1	Part	Power
2	Colour	Greenish Yellow
3	Taste	Bitter
4	Odour	Characteristic Strong odour

b) Physicochemical Properties of *Panchashirisha agada*:

Table no. 6 Represents Physico-chemical properties of *Panchashirisha agada*

SL.NO	TESTS	RESULTS
1	Loss on Drying	6.349%
2	Ash Value	5.640%
3	Acid Insoluble Ash	3.468%
4	Water soluble extract	18.033%
5	Alcohol soluble extract	15.949%
6	pH (5% Solution)	6.70%

B) Preliminary phytochemical screening for organic Components

Methanol extract of *Panchashirisha agada* churna was prepared with cold maceration technique. That was further subjected for Qualitative phytochemical screening.

Table no.7 illustrates the result of phytochemicals in *Panchashirisha agada churna* Methanol extract

SL.NO	TESTS	WATER	ALCOHOL
1	Carbohydrates	Positive	Positive
2	Reducing Sugar	Negative	Positive
3	Monosaccharide's	Positive	Negative
4	Pentose Sugar	Positive	Positive
5	Non reducing sugar	Negative	Negative
6	Hexose Sugar	Positive	Negative
7	Proteins	Positive	Negative
8	Amino acids	Positive	Negative
9	Steroids	Negative	Negative
10	Flavonoids	Positive	Positive
11	Alkaloids	Negative	Negative
12	Tannin	Positive	Positive
Glycosides			
13	Cardiac Glycosides	Negative	Positive
14	Anthraquinine glycosides	Negative	Positive
15	Saponin glycosides	Negative	Negative

D) Antibacterial Activity- Cup Plate Method

Table no. 8 Antibacterial activity Cup plate method *Panchashirisha Agada* (ASE)

	1	2	3	4	5	6	7
Extract added→ Organism	100 µl	75 µl	50 µl	25 µl	10 µl	5 µl	No drug
Staphylococcus aureus	32mm	28mm	20mm	NZ	NZ	NZ	NZ
Streptococcus pyogens	27mm	19mm	14mm	NZ	NZ	NZ	NZ
Pseudomonas aeruginosa	32mm	28mm	25mm	NZ	NZ	NZ	NZ
Escherichia coli	30mm	22mm	15mm	NZ	NZ	NZ	NZ

Abbreviation:*NZ=No Zone of Inhibition around the drug

E) Antibacterial Activity –MIC

Table no. 9 Antibacterial activity of *Panchashirisha agada* (Alcoholic extract)

	1	2	3	4	5	6	7	8	9	10	PC	NC
Extract added→ Organism	200micro L	150	125	100	75	50	25	12	10	5	No drug	No Organism
Staphylococcus aureus	T	T	T	T	T	T	T	T	T	T	T	NT
Streptococcus pyogens	T	T	T	T	T	T	T	T	T	T	T	NT
Pseudomonas aeruginosa	T	T	T	T	T	T	T	T	T	T	T	NT
Escherichia coli	T	T	T	T	T	T	T	T	T	T	T	NT

Abbreviations;*NT-No Turbidity, T-Turbidity

F) Antifungal Activity Cup Plate Method

Table no. 10 Antifungal Activity of *Panchashirisha Agada* Cup Plate Method

Sr. No	Sample	Concentration	Zone of inhibition (mm) *Candida albicans*	Zone of inhibition (mm) *Aspergilus barsiliens*
1	Control	-	-	-
2	Standard Fluconazole	1mg/ml	18	10
3	Sample- ethanolic extract	5mg/ml	02	03
		10mg/ml	14	04

DISCUSSION

- Phytochemical screening shows that, extract of *Panchashirisha agada* has the presence of carbohydrates, flavonoid, tannins and saponin in it.
- Anti-bacterial study revealed that the order of activity against selected bacteria by methanol extract of *Panchashirisha agada* was *Staphylococcus aureus* (32mm), *Streptococcus pyogens* (27mm),

Pseudomonas aeroginosa (32mm), *Escherichia coli*(30mm) for 100µl concentration. The control antibiotic had diameter of inhibition ranging between 36mm to 41mm.

- *Staphylococcus aureus* and *Pseudomonas aeroginosa* were observed as more resistant bacteria with highest inhibition concentration.
- It's been observed that, the methanol extract of *Panchashirisha agada* shows increasing inhibition with increasing concentration of extract.
- The result indicated that bacteria *Staphylococcus aureus, Streptococcus pyogens, Pseudomonas aeroginosa and Escherichia coli*, being negatively affected by low concentration tested from 5µl to 25µl.
- The antibacterial activity of methanol extract of *Panchashirisha agada* on selected pathogen indicate that it has good source of antibacterial capacity against both gram positive and gram negative strains namely *Staphylococcus aureus, Streptococcus pyogens, Pseudomonas aeroginosa and Escherichia coli*.
- Anti-fungal activity study showed the good inhibiting the growth of fungus against *Candida albicans* at concentration 10mg/ml and poor inhibiting growth of fungus against *Aspergilus barsiliensis*.
- Anti-fungal activity study indicated ethanolic extract of *Panchashirisha agada s*howed fungus inhibiting concentration 14mm for *Candida albicans* and 4mm for *Aspergilus barsiliensis* respectively for 10mg/ml concentration. The control antifungal diameter of inhibition is 18mm and 10mm for *Candida albicans* and *Aspergilus barsiliensis* respectively.

CONCLUSION

In spite of having a number of microbial drugs in last 3 decades there is increase in number of anti-microbial drug resistance due to over and misuse of anti-microbial. So it is need of an hour to search new and broad spectrum alternative anti microbial agents. *Acharya charaka* stated *Panchashirish agada* as *vishnampravarovadhe*; considered best in treating all kind of *vishajnya* condition. *Acharya sushruta* mentioned *Panchashirish agada* for treating *keetavisha*.

Anti–bacterial study revel that methanol extract of *Panchashirish agada* possess appreciable anti bacterial activity against *Staphylococcus aureus, Streptococcus pyogens, Pseudomonas aeroginosa and Escherichia coli*. The result of anti-fungal activity indicates that ethanol extract of *Panchashirish agada* is good source of antifungal capacity against *Candida albicans and Aspergilus barsiliensis*.

An anti-microbial activity of *Panchashirish agada* to test organisms as reveled in the study

implies that *Panchashirish agada* could be used in treatment of wide range of bacterial infection.

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