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ANALYTICAL STUDY OF PRATHAPABHAIRAVA RASA AND ITS EVALUATION OF ANTI BACTERIAL ACTIVITY ON *E.COLI* AND *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Introduction: Rasashastra is a branch of Ayurvedic pharmaceutics which utilises metals, minerals, gemstones, animal products and herbal ingredients to formulate medicines which acts as rejuvenative, cures chronic diseases and ultimately helps to increase longeivity. Prathapabhairava rasa is one such Rasoushadhi mentioned in Atisara Adhyaya of the book Rasa Jala Nidhi. **Materials and methods:** Prathapabhairava rasa contains Shudha Hingula, Shudha Gandhaka, Shudha Vatsanabh, Shudha Tankana, Triphala churna and Jambeera swarasa as bhavana dravya. **Results:** Prathapabhairava Rasa had Anti-microbial action against Gram Negative bacteria *E.coli* and Gram Positive bacteria *Staphylococcus aureus* which cause various infectious diseases in human being. By the results obtained through Anti-bacterial study of prepared drug it is proved that Prathapabhairava rasa can be therapeutically more effective.

KEYWORDS: Prathapabhairava Rasa, Rasoushadhi, Anti bacterial.

INTRODUCTION

Infectious diseases are the world's leading cause of premature deaths, killing almost 50,000 people every day. Infections due to variety of bacterial etiologic agents such as pathogenic *Escherichia coli*, *Staphylococcus aureus* are most common.[1] Escherichia coli is a gram- negative bacilli which is one of the most frequent causes of many common bacterial infections, including cholecystitis, bacteremia, cholangitis, urinary tract infection (UTI) and traveller's diarrhoea, and other clinical infections such as neonatal meningitis and pneumonia.[2] *Staphylococcus aureus* is a Gram Positive, round-shaped bacterium and a common cause of skin infections, respiratory infections and food poisoning. It is the most common cause for bacterial diarrhoea. With the continuous use of antibiotics micro-organisms have become resistant. Therefore, there is a need to develop alternative anti-microbial drugs for the treatment of infectious diseases. As mentioned in Rasa Jala Nidhi text, Pratapabhairava Rasa is indicated in Atisara, Kasa, Swasa, Grahani, Sannipataja vikaras, Apasmara, Vataja vikaras, Ajeerna and Agnimandhya disorders. The above mentioned diseases are also caused due to micro-organisams and hence there is a need to study and evaluate this preparation under other modern parameters.

MATERIALS AND METHODS

Pharmaceutical study

The pharmaceutical study was conducted at JSS Ayurveda Medical College, Mysuru.

Prathapabhairava Rasa:[3] Ingredients- Shudha Hingula, Shudha Vatsanabha, Trikatu churna, Shudha Tankana, Shudha Gandhaka, Jambeera Swarasa as Bhavana dravya.

1. Shodhana of Hingula- Hingula was given bhavana with Ardraka swarasa for 7 days.[4]
2. Shodhana of Gandhaka- Gandhaka was melted with equal quantity of Ghritha and was poured into Goksheera. It was repeated for 7 times.[5]
3. Shodhana of Vatsanabha - Small pieces of Vatsanabha roots were soaked in sufficient quantity of gomutra for 3 days by replacing with fresh gomutra for each day. On 4 th day, the Vatsanabha was take out, washed with hot water and the Shudha Vatsanabha was dried completely under hot sun. After complete drying it was powdered.[6]
4. Shodhana of Tankana- Tankana was taken in an iron pan and bharjana was done until water content evaporated.[7]
5. Preparation of Trikatu churna – Shunti, Maricha, Pippali were taken in equal quantity, finelypowdered and mixed well.[8]
6. Preparation of Prathapabhairava Rasa - Equal quantities of Shudha Hingula, Shudha Gandhaka Shudha Vatsanabha, Shudha Tankana and Trikatu churna, were taken in Khalva Yantra. To this sufficient quantity of Jambeera swarasa was added

and mardana was done for two yaama. After 6 hours the product was dried powdered and stored in air tight container.

Analytical Study

The Physico-chemical Analysis of the drug sample was carried out at Ganesh Consultancy and Analytical Services, Mysuru. The Special Instrumental Analysis was conducted at the Vignana Bhavan of Manasagangotri, Mysuru.

Methodology Analytical Study

Modern parameters - Physico-Chemical study (According to API)

a) pH: Procedure: To 10 g of the sample, 100ml of distilled water was added, stirred for 1⁄2 hour, filtered and the pH of the filtrate was noted in a pH paper or pH meter.

b) Ash Value: Procedure: 1gm of the sample was taken in a previously weighed crucible & ignited in an electrical muffle furnace at 450°C. The crucible was cooled and weighed. From the weight of ash obtained, the percentage of ash was calculated and expressed as %w/w.

c) Acid Insoluble Ash: Procedure: The ash was transferred to a 100ml glass beaker & treated with 25ml 6N HCl and the solution was boiled for 5 minutes & filtered through ash less Whatsmann filter paper no.41. The residue over filter paper was collected & dried in an oven at 110°C. The residue along with the filter paper was ignited in a previously weighedcrucible, at 450°C in the electric muffle furnace. Then, the crucible was cooled and weighed. From the weight of the ash the percentage of acid insoluble ash content was calculated. The filtrate obtained was kept separately and it is used for further analysis.

d) Water soluble ash: Procedure: The total ash was taken in 100ml glass beaker & 25ml of water was added, the solution was boiled for 5 minutes & filtered through ash less Whatsmann filter paperno.41. The residue over filter paper was collected & dried in an oven at 110°C.The residue along with the filter paper was ignited in a previously weighed crucible, at450°C in the electric muffle furnace. Then the crucible was cooled and weighed. From the weight of the ash, the percentage of water soluble ash content was calculated.

e) Loss on ignition: Procedure: Silica crucible was weighed previously and ignited for one hour at a temperature not exceeding 500^0C and cool in desiccators. Accurately weighed sample of Prathapabhairava *Rasa* was transferred to the crucible. Weigh the crucible accurately. Loaded crucible was placed in the muffle furnace and ignited to 500 ^{0}C, until constant weight was indicated. Loss on ignition with reference to the air dried drug was calculated.

f) Loss on drying: Procedure: About 10g of drug was placed after accurately weighing in a tarred evaporating dish and dried at 110 ^{0}C for 5 hours, and weighed. Drying was continued and weighed at one hour intervals until the difference between two successive weighing corresponds to not more than 0.25 percent. Constant weight was reached when two consecutive weighing after drying for 30 minutes in a desiccator.

g) Microbial contamination: Procedure: 1g of sample with a 5ml of 0.5% (W/V) sterile saline was taken in a pipette and agitated. The samples were serially diluted in the range of 10-1to 10-8.Agar plating technique by spread plate method was adopted.

h) Particle size: Procedure: Laser Particle Size Analysis method was adopted to determine the particle size.

3. Characterization: Characterization by using SEM, EDAX and FTIR was carried out.

Anti Bacterial Study

The evaluation of Anti-bacterial activity was carried out at Karyome Private Limited, Mysuru- 570026, Karnataka.

Antimicrobial Screening: Agar well diffusion method has been used to determine the antimicrobial activities and resazurin assay for determining the minimum inhibitory concentrations (MIC) of sample against Gram-positive bacteria (*Staphylococcus aureus*),Gram-negative bacteria (Escherichia coli) at a viable count of 106CFU/ml.

Preparation of Agar Medium and Agar Plates: Mueller Hinton Agar 38gm was suspended in 1000ml distilled water and was heated to boiling until the medium dissolved completely. Then it was sterilized in autoclaving at 15 lbs pressure (121^0 C) for 15 minutes and then cooled to 45-50^0C. The medium was mixed well and poured (approximately 20ml) into sterile Petri plates and allowed to cool.

Preparation of Inoculums: A loop of bacterial culture was inoculated into the nutrient broth medium and incubated at 35^0C for 10-12 hours. 1 ml of this culture was used for the experiment.

Procedure: One ml of fresh bacterial was pipetted on Molten cooled Muller Hinton agar. Upon solidification, wells were made using a sterile cork borer (6 mm in diameter) into agar plates containing inoculums. Then, 100 µl(10% & 20% w/v)of the sample was added to respective wells of each plate. The plates were let sit for 30 mins for the extracts to diffuse into the well and was incubated at 37°C for 18 h. Antimicrobial activity was detected by measuring the zone of inhibition (including the wells diameter) appeared after the incubationperiod.

MIC determination by Resazurin assay

Ingredients: Tryptone- 10gm/L, Yeast Extract- 5gm/L, Sodium Chloride- 10gm/L, Agar- Agar- 15gm/L; pH:7.5.

Autoclave the media at 121°C/15lbs.

Procedure: The sterile 96 well plate was labelled. A volume of 50 µL of test material in Luria Bertani broth was pipetted into the first and second row of the plate. In the third row, Ampicillin (100mg/ml) as positive control and to the fourth row Luria Bertani broth was added. The serial dilutions were performed using a multichannel pipette. To each well Resazurin indicator solution at 10 µL was added. Finally, 10 µL of *E. coli* was added to each well to achieve a concentration of 10^6 CFU/mL. The plate was covered with aluminium foil to prevent dehydration and was incubated overnight at 37°C. The change in colour from purple to pink was assessed visually and was recorded positive. The lowest concentration at which colour change is observed is the MIC value for the test material and bacterial strain.

OBSERVATIONS AND RESULTS OF ANALYTICAL STUDY

1. Classical Parameters

Table 1: Organoleptic test.

Sample	Prathapabhairava Rasa
Swaroopa	churna
Sparsha	Smooth, No perceptible coarse particles
Varna	brick red.
Rasa	katu
Gandha	gomutra gandha

2. Modern parameters

Table 2: Physico-chemical tests of *Prathapabhairava Rasa*.

Sl.No.	Parameters	Unit	Results	Test Method
1.	Total ash	%	14.44	General guidelines for drug development for Ayurveda formulations "Central councilfor research in ayurvedic sciences" ministry of ayush government of India
2.	Water soluble ash	%	3.92	
3.	Acid insoluble ash	%	1.47	
4.	Loss on ignition	%	85.56	
5.	Loss on drying	%	9.95	
6.	pH in 10% solution	---	5.17	GCAS/SOP/7.2/F-20
7.	Particle size	Microns	7-126	FSSAI Manual of method

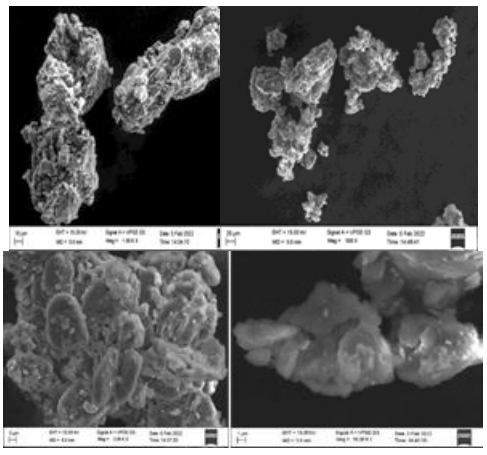
Table 3: Microbial contamination test of Prathapabhairava Rasa.

Sl.No.	Parameters	Unit	Results	Test Method
1.	Total Microbial count	Cfu/g	840	IS5402
2.	Coliform	Cfu/g	<10	IS5401(part1)
3.	*E.coli*	Cfu/g	<10	IS 5887(part 1)
4.	*Staphylococcus aureus*	Cfu/g	<10	IS:5887(part 8 sec 1)
5.	Salmonella	/25g	Absent	IS 5887(part 3)

CFU: ColonyForming Unit.

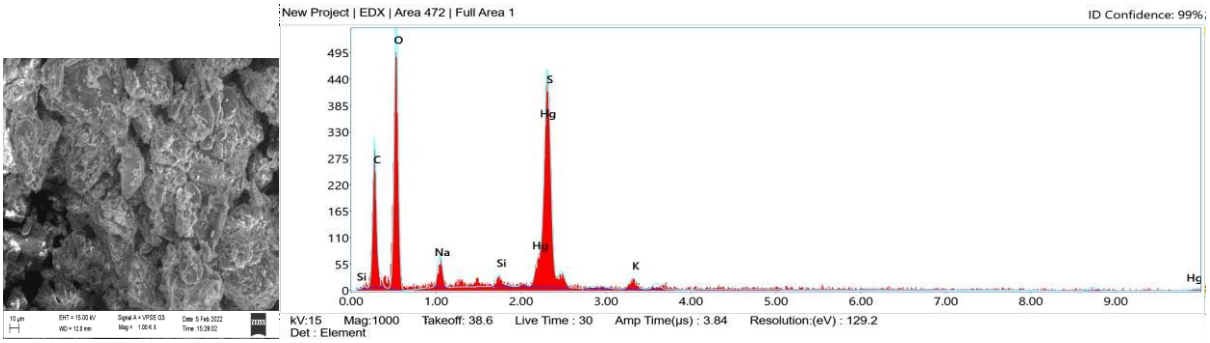
3. Characterization

a) SEM



SEM yielded high resolution figures of the sample surfaces. It showed a characteristic three dimensional appearance which was useful for determining the surface structure of the sample.

b) EDAX

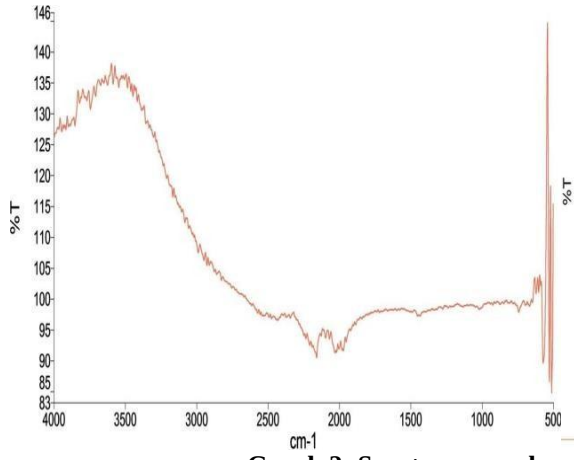


Graph 1: EDAX of Prathapabhairava Rasa.

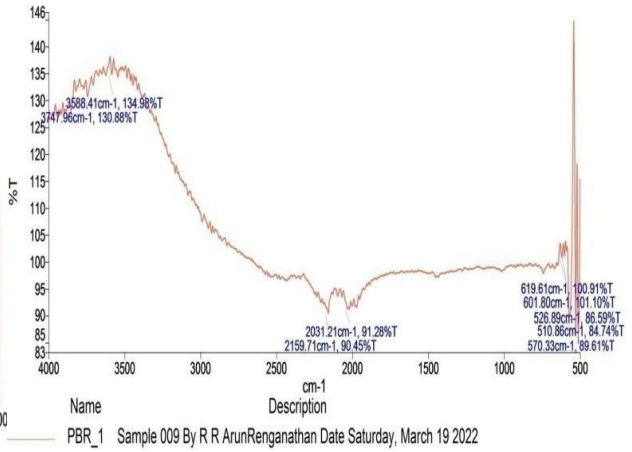
Table 4: Quantitative Results for: Base (466) Prathapabhairava Rasa EDAX.

Element	Weight %	Atomic %
O K	40.2	43.0
C K	30.6	43.6
Na K	3.1	2.3
Si K	0.5	0.3
S K	17.9	9.5
K K	1.7	0.8
Hg M	5.9	0.5

c) FTIR



Graph 2: Spectrum graph.

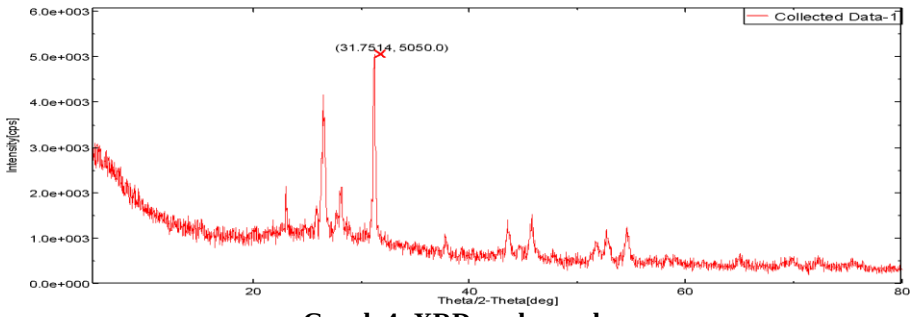


Graph 3: Peak table graph.

Peak table

Peak	X(cm-1)	Y(%T)	Peak	X(cm-1)	Y(%T)	Peak	X(cm-1)	Y(%T)	Peak	X(cm-1)	Y(%T)
1	3747.96	130.88	2	3588.41	134.98	3	2159.71	90.45	4	2031.21	91.28
5	619.61	100.91	6	601.8	101.1	7	570.33	89.61	8	526.89	86.59
9	510.86	84. 74									

d) XRD

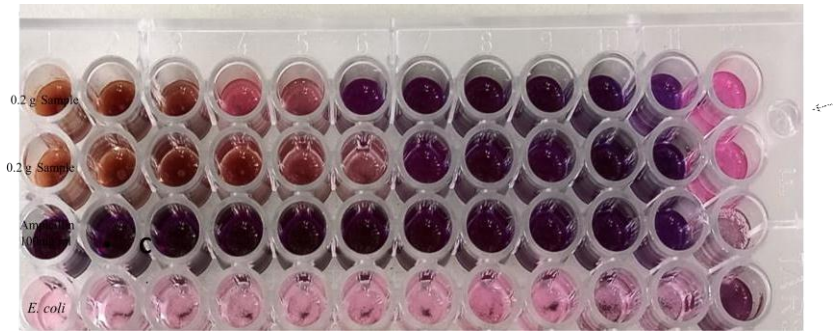


Graph 4: XRD peak graph.

Observations and Results of Anti-Microbial Study
Observations on Antibacterial Activity: By Agar Well Diffusion Method - Antibacterial activity of Prathapabhairava Rasa on *Staphylococcus aureus* and *E.coli* was detected in the agar plates after the incubation period of 18 hours by measuring the zone of inhibition. The zone of inhibition was found nil for Prathapabhairava Rasa at 10mg concentrations for both *Staphylococcus aureus* and *E. coli*. The zone of inhibition was found to be 8mm for *Staphylococcus aureus* and 10mm for *E.coli* at 20mg concentration.

The arrow indicates the zone of inhibition

Resazurin Assay
Results: The 50 µl sample tested by serial dilution showed MIC for *E. coli* inhibition at0.7µg.



DISCUSSION
Analytical Study
pH value (10% solution): pH Value (10% solution) of the drug Prathapabhairava Rasa was 5.17% which indicates that the formulation is highly acidic in nature.

Ash value: The ash value of the preparation is 14.44%, it indicates that the sample is having 14.44% of inorganic matter in total and can be inferred that the rest of 85.56% would be the organic matter present in the formulation which corresponds to the higher proportions of herbal drugs present in the formulation.

Acid Insoluble ash: The acid insoluble ash value of the drug Prathapabhairava Rasa was 1.47%. This indicates the presence of Silica and Siliceous matter present in the formulation which may be from the ingredients of the formulation. Here, this value corresponds only tothe inorganic part. So, 1.47% of the inorganic part of the formulation might not get absorbed.

Water Soluble ash: The Water soluble ash value of the drug Prathapabhairava Rasa was 3.92% which corresponds to the amount of inorganic part of a drug which can dissolve in water. It is very less, which indicates that the drug is soluble in water.

Loss on ignition: Prathapabhairava Rasa was estimated for loss on ignition and it was 85.56%, which indicates that there is maximum loss on heating the drug. But in the body there is not so much temperature that will reduce the efficacy of the drug.

Loss on drying: Prathapbhairava Rasa was found to posses 9.95% loss on drying at 110 ^{0}C hence it can be stated that it posses least moisture and hence very rare chance of bacterial and fungal growth and deterioration chance or contamination chances etc. are very less. Also, it enhances the shelf life of the formulation.

Microbial contamination: The total bacterial count is 840 Cfu/g in this sample. Coliforms, E.coli, *Staphylococcus aureus* count is <10 Cfu/g asit is least count. Salmonella organisms are absent. As the organismos Coliformis, E.coli, Staphylococcus count is <10 Cfu/g and Salmonella organism is absent in the sample there is less or no chances of contamination or growth of bacteria.

Characterization

Particle Size: The particle size of the final product is 7-126 microns; this fineness may bedue to Mardana and Bhavana process done. Smaller particle size increases the surface area of the compound for better action.

SEM: SEM images of the drug Prathapabhairava Rasa showed particles which were not uniform in size and having irregular shape and rough particle surface. It might be due to the presence of herbal drugs like Trikatu churna and Vatsanabha churna. Also, presence of Mercury & Sulphur components and manual handling might have contributed for irregular shape and rough surface of particles.

EDAX: This analysis confirmed the presence of Silicon, Carbon, Oxygen, Sodium, Mercury, Sulphur and Potassium. It showed the presence of Oxygen in 40.2% which was the highest component in the given sample. Presence of Mercury and Sulphur may be because of Hingulaand other inorganic components might be due to herbal drugs used either as the ingredient or for shodhana purpose.

FTIR: FTIR analysis of the sample showed 9 peaks in the mid IR spectrum within a range between 400-4000cm^{-1}.

The mid IR spectrum is again divided into 4 regions:

a) The single bond region (2500-4000cm^{-1})
b) The triple bond region (2000-2500 cm^{-1})
c) The double bond region (1500-2000 cm^{-1})
d) The fingerprint region (600-1500cm^{-1}) The spectra show broad and short peaks.

Two peaks (3747.96cm^{-1} and 3588.41cm^{-1}) lie in the single bond region which rangesbetween 2500-4000cm^{-1}. This indicates the presence of Hydrogen bond, this band confirms the existence of hydrate (H_20), hydroxyl(OH), ammonium or amino groups.

Two peaks (2159.71cm^{-1} and 2031.21cm^{-1}) lie in the triple bond region which ranges between 2000-2500cm^{-1}. These peaks correspond to the presence of thiocyanate and isothiocyanate classes of compounds.

Peaks are absent in the double bond region.

Two peaks (601.8 cm^{-1} and 619.61cm^{-1}) lie in the fingerprint region which ranges between 600-1500cm^{-1}.

On the basis of the obtained data from FTIR analysis, it can be interpreted that the prepared sample has complex chemical structure molecules which are bound strongly.

XRD: The XRD report represents the elements with prominent peaks at 31.7theta around 28^0 and 22^0. This indicates that it is crystalline in nature and insoluble with associated organic materials.

DISCUSSION ON ANTI BACTERIAL STUDY

The zone of inhibition was found nil for Prathapabhairava Rasa at 10mg/ml concentrations for both *Staphylococcus aureus* and *E.Coli* and the same was found to be 8mm for *Staphylococcus aureus* and 10mm for *E.coli* at 20mg concentration. Hence, it can be concluded that the drug is more potent on *E.coli* at 20mg concentration.

The 50 µl sample subjected to serial dilution showed MIC for *E. coli* inhibition at 0.7µg.

CONCLUSION

In the present era due to extensive use of antibiotics and vast majority of synthetic drugs, numerous multidrug resistant strains are developing. Therefore, to overcome drug resistance and to avoid side effects associated with the commonly available antibiotics, there is a surge of another treatment. The alternate management can be achieved by the use of traditional medicinal herbs, metals and minerals which are potent antibacterial agents, clinically safer, cost effective and affordable. Prathapabhairava Rasa is a Herbo-mineral classical Khalvi Rasayana preparation. Analytical study shows that it contains lesser particle size which indicates the greater chances of absorption. The drug also showed the inhibition of *Staphylococcus aureus* and *E.coli* organism considering lowest drug concentrations at 20mg (100µl) in Agar well diffusion method. The MIC determined by Resazurin assay method for Prathapabhairava Rasa on *E. coli* was found to be 0.7µg.

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