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Research Protocol

Anti-Cancer

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A Study Protocol for Pharmaceutico-Analytical study of Rajat Sindoor and evaluation of its Anti-Cancer Activity against Leukaemia using HL60 and K562 cell line model

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Introduction: Leukaemia is a disease of haematological malignancies that result from the abnormal multiplication of developing leukocytes. A patient suffering through leukaemia experiences an aberrant blood cell synthesis, usually involving leukocytes (white blood cells). Leukaemia is categorized into two groups, based on the cell types that are mostly involved: Lymphoid and Myeloid, also based on the disease's natural course: Acute and Chronic. Neoplasms and Vyadhi Arbuda, which is mentioned in the classics of Ayurveda (Charaka Samhita and Sushruta Samhita), are comparable. Rasa Aushadhis have many therapeutic advantages in many chronic health aspects such as CKD, Cardiac conditions, Inflammatory conditions, bleeding disorders, cancers, etc. Rajat Sindoor is one of the effective Rasa Kalpas found no previous work on in-vitro investigation to assess the anti-cancer activity w. s. r. to Leukaemia.

Materials and Methods: Rajat Sindoor will be prepared as per classical reference. The prepared formulation will be assessed with organoleptic characters and physio-chemical parameters. The Experimental study will be carried out according to the OECD guidelines.

Result: The analytical parameters will be assessed to establish pharmaceutical standardization. The experimental study and statistical analysis will be done accordingly.

Discussion: The purpose of the current study is to evaluate Anti-cancer activity of Rajat Sindoor w. s. r. Leukaemia using HL60 and K562 cell line model. A small step to fill the research gap in the treatment aspect of Leukaemia from Ayurvedic point of view will be done. If significant positive results are obtained in this work, then it will be a valuable contribution in the treatment of leukaemia.

Keywords: Leukaemia, Anti-cancer activity, Rasaushadhi, Kupipakwa Rasayana, Rajat Sindoor, HL60 cell line, K562 cell line, Study Protocol.

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Introduction

Ayu is the symbol for life*, and Veda* for knowledge*. Ayurveda* represents knowledge of life which deals with health and also cures diseases. *Rasa shastra* deals with the pharmaceutics and therapeutics of drugs having minerals and metal origin.**[1]** In *Rasa shastra, Rasa* means Mercury. The primary focus of *Rasa Shastra* is Mercury, other Metals, minerals and their pharmaceutical preparations.**[2]** These formulations are prepared by various different methods and procedures mentioned in *Rasa-Granthas* and Ayurvedic texts. One of those techniques *Kupipakwa Rasayana* is a special pharmaceutical technique for making medication, in which the medication is made in a glass bottle known as a *Kachakupi.* The processing is done either in traditional *Valuka Yantra* or in electric muffle furnace with a pattern of gradual rise in temperature.**[3]** *Kupipakwa Rasayanas* are prepared - with Mercury - having two types - *Sagandha* (with Sulphur) and *Nirgandha* (without sulphur).**[4]**

A further valuable metal, silver has a significant role in Ayurvedic medicine. Various useful formulations of '*Rajata*' have following properties: *Balya* (Strength enhancer), *Rasayana* (Rejuvenation), *Medhya* (memory booster), *Ayushya* (betterment of life), *Ojo Vardhaka* (Immunity booster), *Vayah Sthapaka* (Anti-aging properties) etc. **[5]**

Leukaemia is a disease of haematological malignancies that result from the abnormal multiplication of developing leukocytes. A patient suffering through leukaemia experiences an aberrant blood cell synthesis, usually involving leukocytes (white blood cells).**[6]** Leukaemia is categorized into two groups, based on the cell types that are mostly involved: Lymphoid and Myeloid, also based on the disease's natural course: Acute and Chronic.**[7]** We can corelated Leukaemia with the symptoms of *Raktarbuda* mentioned in Ayurvedic literature.

Prevalence of Leukaemia

Cancer has grown to be a significant challenge and source of concern for biologists and medical professionals due to issues with therapy as well as the disease's rising global incidence.**[8]**

In India and throughout the world, leukaemia—of which >95% are acute—represent the most common diagnostic category for paediatric cancers.

Notable advancements have been achieved in the therapy. Of acute lymphoblastic leukaemia (ALL), which accounts for 75–80% of juvenile acute leukaemia, 90% of patients in high-income countries (HICs) survive for five years or more.

Although not particularly noteworthy, advances in acute myeloid leukaemia (AML) have been consistent, with 5-year overall survival rates close to 70%. Longitudinal data from India regarding trends in childhood cancer survival are scarce.

However, published data indicates that there has been improvement in childhood ALL outcomes. However, India has seen more moderate improvement overall. There is not enough information about AML to draw any significant conclusions.**[9]**

Review of Leukaemia

Leukaemia is A clone of malignant cells derived from myeloid or lymphoid stem cells.**[10]**

In children 85% of leukaemia are of lymphocytic type. In adults, acute myeloid leukaemia is the most prevalent kind.

Types of Leukaemia: There are 2 types of Leukaemia

Table 1: Types of Leukaemia

1.	Acute [11,12]	Acute Lymphoblastic Leukaemia (ALL)
		Acute Myeloid Leukaemia (AML)
2.	Chronic [13]	Chronic Lymphocytic leukaemia (CLL)
		Chronic Myeloid Leukaemia (CML)

Common sign and symptoms of leukaemia - [14]

- Anaemia - pallor, tiredness, Cardiorespiratory symptoms
- Granulocytopenia - Fever (flu like symptoms)
- Thrombocytopenia - results in bleeding
- Hyperleukocytosis
- Hepatomegaly / splenomegaly

Review of *Raktarbuda*

Acharya Sushruta explained *Raktarbuda* briefly in *Sushruta Samhita's Nidansthana***[15]** and *Chikitsasthana*.**[16]** If we look at pathophysiology and common signs and symptoms of leukaemia, we can compare this disease as follows **[17]** –

Table 2: Comparison between symptoms acc. to Modern and Ayurveda.

Signs and symptoms acc. to modern	Lakshanas / conditions acc. to Ayurveda
Anaemia	Pandu
Pallor	Pandurata
Tiredness	Tandra
Cardiorespiratory symptoms	Shwasa
Fever	Jwara
Thrombocytopenia (Bleeding)	Raktapitta
Hepatomegaly	Yakrutodara
Splenomegaly	Pliha Vriddhi
Enlarged lymph glands	Granthi / Arbuda

Review of *Rajat Sindoor*

"*Rajat Sindoor*" is mentioned in *Rasendra Sambhav.* **[18]**

In Ayurvedic classical texts many *Kupipakwa Rasayanas* have been explained, *Rajat Sindoor* is one among the Herbo-mineral formulations (rational combination of *Rasadravyas* and *Kashthaushadhi*).

It is also mentioned as *Kupipakwa Rasayana* in classical reference - *Ayurved Sar Sangraha.* **[19]**

It contains *Shuddha Parada*, *Shuddha Gandhak*, *Shuddha Rajat*, *Kumari Swaras.*

Previous Work Done

As per the available literature review, no previous work was found on Pharmaceutico-Analytical study of *Rajat Sindoor* and evaluation of its anti-cancer activity against Leukaemia (In vitro study). Although, various work has been done on Leukaemia and Ayurvedic approach, Ayurvedic management of Leukaemia, Standardization of *Rajat Sindoor* and also on its analytical assessment.

Nature of Knowledge Gap Identified –

Table 3: Knowledge Gap

Research Gap Type	Type of research gap used
Knowledge Gap	Preparation of Rajat Sindoor using ratio of 4:4:1 for raw material is rarely used.
Methodological Gap	After extensive literature search, the research scholar could not find any Anticancer study of Rajat sindoor w.s.r. Leukaemia.
Empirical knowledge gap	Insufficient literature regarding conceptual, pharmaceutical, analytical studies has formed an empirical knowledge gap.

Need of Study

- According to the data shared by Globocan 2022, the incidence of leukemia across the world ranks 13th place among all types of cancers, whereas the mortality data states that it ranks 10th place.**[20]**
- According to the report of Globocan 2022, Incidence of Leukemia in India ranks 8th place among all types and the mortality data states its 9th place. This indicates that 4% of deaths occurred due to leukemia.**[21]**
- According to research conducted by population-based cancer registries of India, in 2010, projected number of lymphoid & haematopoietic system (LHS) cancer cases was 41,591 for females & 62,648 for males. It is anticipated that by 2020, there will be 55,384 instances for females & 77,190 cases for males. Myeloid leukaemia (34,701, 26.2%) & NHL (38,098, 28.7%) are predicted to have largest contributions to LHS-related cancers in 2020.
- To create awareness regarding treatment for leukaemia and also to contribute and validate *Rajat Sindoor* as treatment of leukaemia.
- The clinical co-relation of leukaemia with ayurvedic diagnosis, its pathophysiology is needed.

Aim and Objectives

Aim

To study pharmaceutico-analytical aspects of *Rajat Sindoor* and evaluation of its anti-cancer activity against leukaemia using HL60 and K562 cell line model.

Objective

Primary objectives:

1. To study pharmaceutical process of *Rajat Sindoor*.
2. To study analytical parameters of *Rajat Sindoor*.
3. To evaluate anticancer activity of *Rajat Sindoor* against Leukaemia using HL 60 cell line model.
4. To evaluate anticancer activity of *Rajat Sindoor* against Leukaemia using K562 cell line model.

Secondary objectives:

1. To study the effect of *Rajat Sindoor* on cell death mechanism.

2. To study the anti-oxidant properties of *Rajat Sindoor.*

Materials and Methods

Present work will be conducted under following headings

A) Pharmaceutical Study:

Procedure for preparation of *Rajat Sindoor* will be carried as mentioned in *Rasendra Sambhav.*

Ingredients of *Rajat Sindoor*

Table 4: Contents of *Rajat Sindoor*

Ingredients	Latin name	Part used	Proportion of Ingredients
Shuddha Parad	Hydrargyrum (Hg)	Pure Mercury	4 parts
Shuddha Gandhak	Sulfurium (S)	Pure Sulphur	4 parts
Shuddha Rajat	Argentum (Ag)	Pure Silver	1 part
Kumari Swaras	Aloe vera	Leaves juice	Q. S.

Method of preparation of *Rajat Sindoor* [22]-

Collection of raw materials (*Parad, Gandhak, Rajat*)

↓

Shodhana (purification) of *Parad, Gandhak.*

↓

Samanya Shodhana of *Rajat* and *Vishesh Shodhana* of *Rajat.*

↓

Amalgamation of Purified silver and purified mercury.

↓

Preparation of *Kajjali* with fine powder of sulphur and amalgam of silver and mercury.

↓

The process of *Bhavna* (trituration) by *Kumari Swaras* (juice of aloe vera leaves) to *Kajjali.*

↓

Kajjali filling in the *Kupi* (glass bottle) and heating *Kupi* in the electric muffle furnace to prepare *Rajat Sindoor.*

↓

Collection of *Rajat Sindoor* from neck region of bottle after self-cooling by breaking the bottle.

B) Analytical Study:

Analytical work of prepared *Rajat Sindoor* will be carried out at a government authorized laboratory.

Raw material analysis, In-process analysis, Analysis of prepared *Rajat Sindoor* will be carried out.

C) Experimental (in-vitro) Study:

1. **HL60** [cell culture model of acute myeloid leukaemia (AML)]**[23]** and **K562** [cell culture model of chronic myeloid leukaemia (CML)] **[24]** cell culture.
2. Measurement of Cytotoxicity by MTT Assay of *Rajat Sindoor*.
3. Determination of Anti-oxidant properties of *Rajat Sindoor* by DPPH scavenging activity.
4. Determination of cell apoptosis mechanism using *Rajat Sindoor* by flow cytometry.

Methods

Pharmaceutical Study

***Parad Samanya Shodhana* (Purification) [25]**

- Various methods of *Parad Shodhana* are mentioned in different *Rasa Granthas*. Among those reference*, Parad Shodhana* with *Rason* (Garlic) is mentioned in *Parad Samhita*.
- *Rason* (garlic) is the antidote for heavy metal poisoning specially for mercury as it contains sulphur and hence an innate co-drug for *Parada Bandha*.
- *Rason* will be crushed and paste of *Rason* will be prepared. *Mardana* of *Parad* with paste of *Rason* will be done until it becomes black in colour.
- This black product will be washed with lukewarm water to collect *Shodhit Parada.*

***Gandhak Shodhana* (Purification)[26]**

- A wide mouthed stainless-steel container will be taken with warm cow's milk in it.
- Tie a cotton cloth as it will cover mouth of container containing milk.
- Required quantity of *Gandhaka* will be melted (120°C-150°C) using Cow's ghee in container with medium flame.
- Pour molten *Gandhak*into milk. Once it solidifies, remove *Gandhaka* & wash it with warm water to remove stickiness of ghee & milk.
- Repeat this procedure of *Shodhana* 3 times.
- Thus, *Shodhit Gandhaka* will obtain by this method of *Gandhaka Shodhana.*

***Samanya Shodhana* (Purification) of *Rajat*[27]**

- *Ashuddha* sheets of Silver will be taken & kept on gas flame with help of iron tongs, heated till it becomes red hot. Red hot sheets then quenched in *Til* tail (Sesame oil) (which will be kept in stainless steel container) until sheets get self-cooled.
- Repeat the procedure for 6 more times. The process of heating the sheets and quenching will be repeated with *Takra* (buttermilk), *Gomutra* (cow's urine)*, Kanji* (fermenting rice porridge)*, Kulattha Kwath* (Horse gram (Macrotyloma uniflorum) infusion) in sequential order, each for 7 times. After completion of *Samanya Shodhana* process, *Rajat* sheets will be washed with water.

***Vishesh Shodhana* (Purification) of *Rajat*[28]**

- *Samanya Shodhit* sheets of *Rajat* will be kept on gas flame with the help of iron tongs until it becomes red hot.
- Red hot sheets then quenched into *Nimbu Swarasa*.
- Repeat the procedure for 3 times. *Shodhit* sheets will become dull and brittle after completion of *Shodhana*.

Preparation of *Kajjali*

- Amalgamation of *Shodhit Parad* and *Shodhit Rajat* will be done by adding molten *Rajat* in *Shodhit Parad* in a *Darviyantra* (table spoon of bigger size).
- *Kajjali* will be prepared by adding *Shodhit Gandhaka* in the amalgamation of *Rajat* and *Parad* in a *Khalwa Yantra* (pestle and mortar).
- Thus*, Kajjali* will prepare.
- The process of *Bhavna* by *Kumari Swaras* (juice of aloe vera leaves) will be done to prepare *Kajjali* until it soaks all the juice.

Preparation of *Rajat Sindoor*[29]

- Prepared *Kajjali* having *Nishchandratva* will be added inside a *Kachakupi* upto 1/3rd of its length. *Kachakupi* is covered with 7 layers of mud smeared cloth.
- This *Kajjali* filled *Kachakupi* will be kept in the electric muffle furnace.
- *Kupi* will be subjected to gradual heat. Temperature pattern (*Dipagni* - Room temp to 100°C*, Mrudu Agni* – 100°C to 250°C*, Madhyamagni* – 250°C to 450°C*, Tivragni* – 450°C to 650°C*, Swangshitikaran* – self cooling) will be maintained as mentioned in classics. This should be in increasing order.
- After complete cooling, *Kupi* will be removed from EMF. Bottle will be broken into 2 halves and *Sindoor* will be collected from neck region of *Kupi* and store in a container.
- *Rajat Sindoor* will be prepared into 3 batches. Analytical study of best prepared batch will be performed.

Analytical Study

Descriptive - Subjective par. of finished product - (Organoleptic Characters) and Objective parameters of finished product (Physiochemical parameters)

Table 5: Analytical parameters

Organoleptic	Physio-chemical test
Colour	pH
Odour	Water soluble ash
Taste	Acid In soluble ash
Texture	Total ash value
Weight	Loss on Drying
	XRD[30]
	SEM-EDAX

Experimental Study
Intervention

1) Study of Cytotoxic effect of *Rajat Sindoor* on HL60 and K562 cell line model by using MTT ASSAY:[31]

- MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] is a pale-yellow substrate that will cleave by living cells to yield a dark blue formazan product.

- This process requires active mitochondria, and even freshly dead cells do not cleave significant amount of MTT.
- Thus, amount of MTT cleaved is directly proportional to number of viable cells present, which will be quant. by colorimetric methods.
- This assay will be performed using the standard operating procedures.
- Briefly the compounds will be dissolved in DMSO and serially diluted with complete medium to get the concentrations a range of test concentration. DMSO concentration will be kept < 0.1% in all the samples HL60 and K562 cells maintained in appropriate conditions will be seeded in 96 well plates and treated with different concentrations of the test samples and incubated at 37 °C, 5% CO2 for 96 hours.
- MTT reagent will be added to wells and incubated for 4 hours; dark blue formazan product formed by cells will dissolve in DMSO under safety cabinet and read at 550nm.
- **Percentage inhibitions will be calculated and plotted with the concentrations used to calculate the IC50 values.**

2) Study of Cell death mechanism by Flow cytometry:[32]

- HL60 and K562 cells maintained in appropriate conditions will be treated with the test samples 10uM for the given time period 4 hours.
- Cells will trypsinize & wash with PBS twice.
- The single cell suspension will be fixed with methanol and stained with propidium iodide.
- The sample will be analysed on a BD FACS calibre flow cytometer.

3) Study of Anti-oxidant properties of *Rajat sindoor* by DPPH scavenging activity:

- DPPH scavenging activity of given sample against stable 2, 2 diphenyl 2 picryl hydrazyl hydrate (DPPH) will be determined by the slightly modified method of Brand-W.
- DPPH reacts with an antioxidant compound, which can donate hydrogen, and reduce DPPH.
- Change in colour (from deep violet to light yellow) will be measured at 517 nm on BMG Fluostar (GERMANY) and % inhibition data will be analysed on MARS software BMG (Germany).
- The solution of DPPH in methanol 6 × 10-5 M will prepare fresh daily before UV measurements.
- 3 ml of this solution will be mixed with 100 microgram/ml concentration of given sample.
- The samples will keep in the dark for 15 minutes at room temperature and the decrease in absorbance will be measured.

Specification of instruments and related measurements:

Table 6: List of instruments

Pharmaceutical work	Analytical work	Experimental work
Stainless steel vessel	Platinum / silica dish	Medium
Khalvayantra (pestle and mortar)	pH meter	96 well plates
Spoons	Micropipette	Spectrophotometer
Cotton cloth, Gas cylinder	Weighing machine	BD FACS calibre flow cytometer
Measuring cylinder	Glass plate	
Darviyantra (table spoon of bigger size)	Nessler cylinder	
Kachakupi (Glass bottle)		
Furnace		

Data Analysis:

Data analysis will be done by standard computer program.

Data will be coded and entered in MS Excel work sheet and other statistical software will be used.

Statistical analysis:

1) Results will be expressed in mean value and standard error of the mean.
2) Statistical analysis will be applied accordingly.

Study Setting:

- Pharmaceutical work will be carried in the department of *Rasashastra* & *Bhaishajya Kalpana* of our institute.
- Analytical work will be carried out at a government authorized laboratory.
- Experimental work will be carried out at an authorized research lab.

Duration of study: The duration of study will be 18 months.

Method of selection of study unit

As per standard protocol, the in-vitro study will be done.

A) Inclusion Criteria: Leukaemic cell line HL60 and K562 for studying Anti-cancer activity of *Rajat Sindoor.*
B) Exclusion Criteria: Cell line other than HL60 and K562 will be excluded from this study.

Expected Results / Outcomes

Stepwise observations will be recorded and presented in the form of tables, charts and pictures, etc.

The study findings may show significant anti-cancer activity of *Rajat Sindoor* against HL60 or / and K562 cell line model.

The study's positive findings will demonstrate the cell death mechanism due to *Rajat Sindoor* against HL60 or / and K562 w. s. r. to Leukaemia.

With the positive outcome of *Rajat Sindoor*, we can study further for its in vivo studies as well as clinical studies for large population.

Discussion

The foundation of Ayurveda, *Rasaushadhis* are the key formulations in Ayurvedic treatments. The *Ras* preparation at a little dosage enables it to enter the deepest tissues by slipping via the tiny body channels (*Srotas*). *Rasaushadhi's* potency, duration, and effectiveness are its primary benefits. They often have no taste or smell, only need a tiny dosage to have a powerful, long-lasting effect, and are not required over an extended period of time.

Rasa Aushadhis have many therapeutic advantages in many chronic health aspects such as CKD, Cardiac conditions, Inflammatory conditions bleeding disorders, cancers, etc. Silver has a significant role in Ayurvedic medicine.

Various useful formulations of '*Rajata*' have following properties: *Balya* (Strength enhancer), *Rasayana* (Rejuvenation), *Medhya* (memory booster), *Ayushya* (betterment of life), *Ojo Vardhaka* (Immunity booster), *Vayah Sthapaka* (Anti-aging properties) etc. However, no previous work was found on in vitro investigation to assess the anti-cancer activity of *Rajat Sindoor* w.s.r. to Leukaemia.

Conclusion

For a future literature review, it will be a valuable addition to the conceptual assessment of *Rajat Sindoor*. Standard operating procedures (SOP) for the *Rajat Sindoor*, covering pharmaceutical standardization, will be provided by this study. This is a sincere endeavour towards bridging the gap in the few scientific conclusions that would be gained via analytical research and experiments. The current study aims to evaluate anti-cancerous efficacy of *Rajat Sindoor* in HL60 & K562 cell line model w. s. r. to Leukaemia. It will be a significant addition and validation that *Rajat Sindoor* is acceptable to take for an extended period of time at therapy dosages outlined in the classics.

Scope of study

Pre-clinical research will be advantageous for presenting additional evidence for clinical trials of *Rajat Sindoor.*

Quantitative elemental analysis will establish the benchmark for evaluating this product's quality and be useful in determining its pharmacokinetic and pharmacodynamic properties.

Consent: It is not applicable

Ethical Approval: Ethical approval has been taken from Institutional Ethics Committee at our Institute. Experiments will be carried out at certified Research laboratory after obtaining permission.

Competing interests: No competing interests exist.

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