



ISSN (O): 2320-5407
ISSN (P): 3107-4928

Journal Homepage: www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)

Article DOI:10.21474/IJAR01/24115
DOI URL: <http://dx.doi.org/10.21474/IJAR01/24115>



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ADVANCED RESEARCH (IJAR)
ISSN: 2320-5407
Journal homepage: <http://www.journalijar.com>
Journal DOI:10.21474/IJAR01

RESEARCH ARTICLE

PHARMACEUTICO-ANALYTICAL STANDARDIZATION AND IN-VITRO ANTI-LUNG CANCER ACTIVITY OF SHADGUNABALIJARITARASA SINDOOR AGAINST A-549 HUMAN LUNG ADENOCARCINOMA CELL LINE

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Manuscript Info

Manuscript History

Received: 04 July 2026

Final Accepted: 08 August 2026

Published: September 2026

Key words:-

Shadgunabali jarita Rasa Sindoor, Rasashastra, Kupipakwa Rasayana, Pharmaceutical standardization, XRD, XRF, A-549 cell line, MTT assay, Cytotoxicity.

Abstract

Background: Lung cancer is one of the most common causes of cancer associated mortality worldwide and limits of conventional therapy has resulted in interest in traditional medicinal formulations. Shadgunabali jarita Rasa Sindoor is one of the classical Kupipakwa Rasayanapreparations used traditionally for different diseases but its pharmacological standardisation and anticancer potential needs scientific substantiation.

Objective:

1. To standardize Shadgunabali jarita Rasa Sindoor in classical ayurvedic procedures.
2. To study its pharmaceutico-analytical features
3. To evaluate its in vitro cytotoxic effect on A-549 human lung cancer cell line.

Materials and Methods: The formulation was manufactured by classical processes of Rasashastra after due purification and pharmaceutical processing. The final product was investigated for pharmaceutico-analytical parameters including instrumental characterisation. Cytotoxic activity against A-549 cells was tested by MTT assay. Cell viability and IC-50 values were established.

Results: The standardised preparation was found to be a bright Red Rasa Sindoor with good pharmaceutical properties. XRD verified the existence of crystalline mercury sulphide (HgS) in the cinnabar phase while XRF showed mercury and sulphur as the primary ingredients. The formulation showed concentration dependent cytotoxicity against A-549 cells with IC50 value of 76.41 µg/mL along with typical morphological alterations.

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Conclusion: The Shadgunabali jarita Rasa Sindoor was effectively standardised and analytically characterised. The formulation showed potential in-vitro cytotoxic efficacy against A-549 lung cancer cells and urge additional mechanistic, pre-clinical and clinical investigations.

Introduction:-

Despite considerable breakthroughs in early detection, targeted therapy and immunotherapy, lung cancer remains one of the major causes of death from cancer worldwide. The prognosis of advanced disease is poor because of late diagnosis, tumour heterogeneity, therapeutic resistance and side effects of the treatment. These constraints have generated interest in the identification of safer and more effective therapeutic agents including those originating from traditional systems of care.(1)

Natural products have played a major role in the discovery of anticancer drugs and many essential anticancer drugs in clinical use are of plant origin. Ayurveda, the traditional system of Indian medicine, mentions a large number of herbal and herbo-mineral preparations with therapeutic potential. In its specialised branches, Rasashastra uses pharmaceutical techniques like Shodhana, Jarana, Bhavana and controlled heating to convert raw ingredients into therapeutically acceptable formulations. It is necessary that these preparations are scientifically standardised, thus assuring their quality, reproducibility and safety.(2)

Shadgunabali jarita Rasa Sindoor(3) is a typical Kupipakwa Rasayana(4) made from refined mercury and sulphur by repeated pharmaceutical processing. Such pharmacological changes have been credited with improved therapeutic capabilities in the classical literature, but systematic scientific evidence on its pharmaceutical standardisation, analytical features and biological activity is scarce. Modern analytical techniques such as X-ray diffraction (XRD) and X-ray fluorescence (XRF) and in-vitro biological assays are useful instruments for the assessment of the physicochemical features and biological potential of these formulations.(5)(6)

Hence, present study was undertaken to prepare Shadgunabali jarita Rasa Sindoor as per classical Ayurvedic pharmaceutical procedures, perform pharmaceutico-analytical standardisation using modern analytical techniques and to evaluate its in-vitro cytotoxic activity against human lung adenocarcinoma (A-549) cell line.(7) The present investigation is aimed to create scientific data in support of the quality, reproducibility and preliminary anticancer potential of this historical Rasashastra formulation.

Materials and Methods:-

Study Design:-

The present experimental work was undertaken to investigate Shadgunabali jarita Rasa Sindoor by integrated pharmaco-analytical and biological studies.

The study was carried out in three sequential phases:-

- (i) pharmaceutical preparation of Shadgunabali jarita Rasa Sindoor as per the classical principles of Rasashastra,
- (ii) pharmaceutico-analytical characterisation of the prepared formulation by classical and modern analytical techniques and
- (iii) evaluation of in-vitro cytotoxic activity against the human lung adenocarcinoma (A-549) cell line using MTT assay(8). The complete workflow of the investigation is presented in Figure 1.

Procurement and Authentication of Raw Materials:-

The pure mercury (Shuddha Parada)(9) and purified sulphur (Shuddha Gandhaka)(10) were obtained from the recognised sources and tested for their organoleptic characters before usage. The processing of pharmaceuticals like Shodhana, Kajjali preparation(11) and the other pharmaceutical procedures were undertaken as per the established protocols given in the classical Rasashastra literature.

Raw material collection:-**Pharmaceutical Preparation of Shadgunabalijarita Rasa Sindoor(12):-**

The preparation was done in the Department of Rasashastra and Bhaishajya Kalpana by classical manner of Kupipakwa Rasayana.

Table 1. Pharmaceutical Processing of Raw Materials

Sr. No.	Pharmaceutical Process	Classical Reference	Initial Weight (g)	Final Weight (g)
1	Shodhana of Parada	Parada Samhita(13)	108	105
2	Shodhana of Gandhaka	Rasatarangini	614	602

3	Preparation of Kajjali	Rasatarangini	—	675
4	Bhavana of Kajjali(12)	—	675	685

Purified mercury and purified sulphur were triturated to give uniform lustreless black Kajjali. ShadgunabalijaritaKajjali was prepared by doing later Jarana with sulphur and Bhavana with KumariSwarasa for 6 cycles. The prepared Kajjali was filled in seven layered Kacha Kupi and heated in Valuka Yantra/ EMF under controlled Mridu, Madhyama and Tivragi. Classical pharmaceutical observations were made during the heating process such as colour changes, sulphur emissions, features of the flame and product deposition. The bright red crystalline Rasa Sindoor recovered at the neck of the bottle after self-cooling was pulverised and stored in sealed glass containers for further analytical and biological examination.

Purification of Parad:-



Purification of Gandhak:-**Formation of Shadgunabalijarita Kajjali:-****Bhavana To Prepared Kajjali:-**

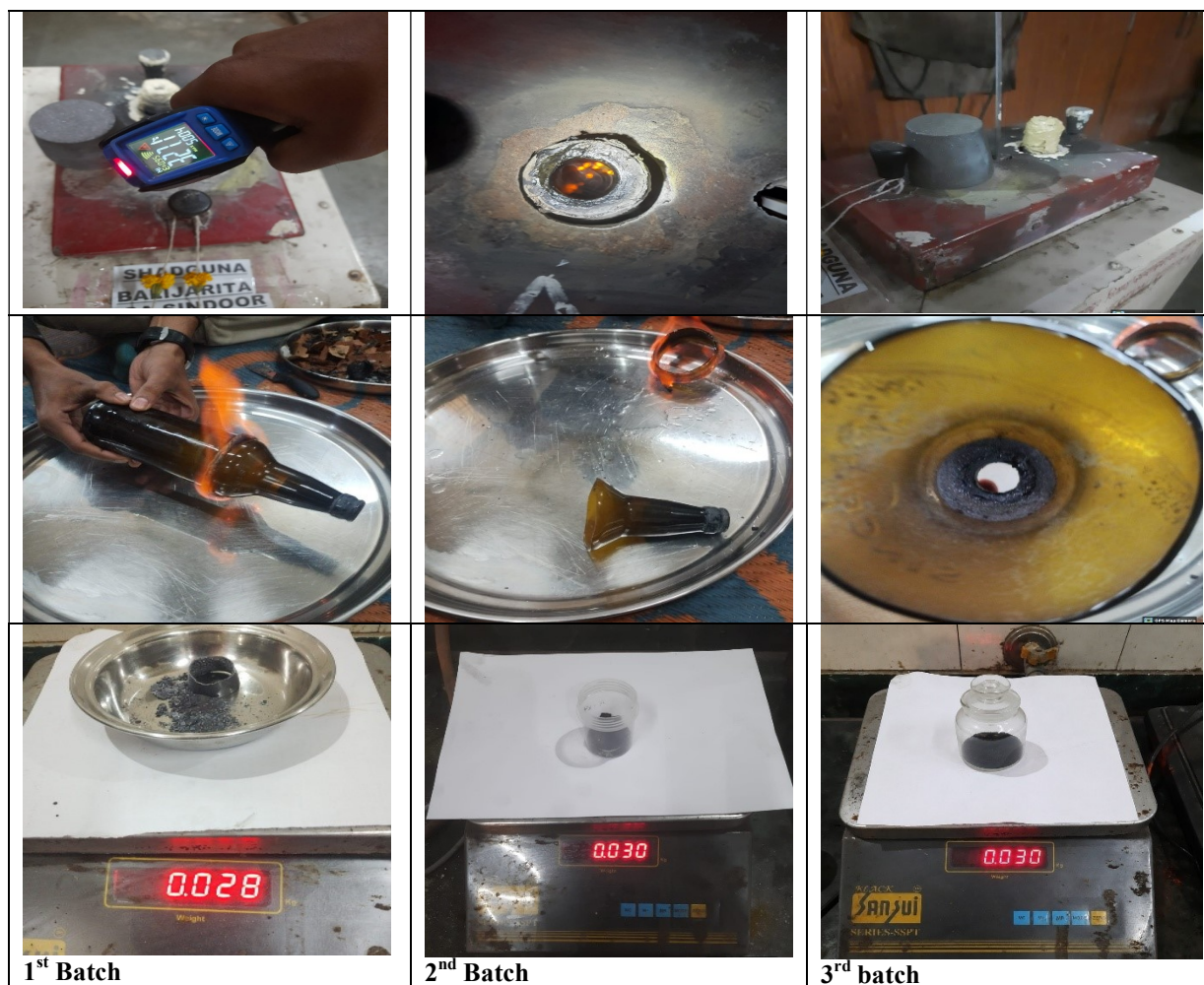
Table 2: Comparative Batch wise pharmaceutical preparation of Shadagunabali jarita Rasasindoor

Time (hrs)	Batch-I (Temp °C / Observation)	Batch-II (Temp °C / Observation)	Batch-III (Temp °C / Observation)
0	50°C (Accel. 5) – Kupi started	50°C (Accel. 5) – EMF started	50°C (Accel. 5) – EMF started
2–3	110°C – Puffiness in Kajjali, Gandhaka smell	120°C – Puffiness of Kajjali	120°C – Puffiness of Kajjali
4	160°C – Kajjali melted, yellow fumes	160°C – Yellow fumes with smell	160°C – Yellow fumes with smell
6	200°C – Yellow fumes with smell	183°C – Dense yellow + white fumes	183°C – Dense yellow + white fumes
10	250°C – Kajjali started melting, dense yellow fumes	260°C – Dense yellow fumes, neck deposition	260°C – Dense yellow fumes, neck deposition
14	320°C – Dense yellow fumes, bottom not visible	323°C – Slight golden-orange base, fumes continue	323°C – Slight golden-orange base, fumes continue
16–18	360°C – Blue flame during Hot Shalaka Sanchalana	350°C – Golden-red base visible	350°C – Golden-red base visible
20–22	387°C – Kajjali boiling, yellow fumes	390°C – Reddish-orange base with fumes	390°C – Reddish-orange base with fumes
24–26	410–425°C – Golden-red base, neck deposition	407°C – Kajjali boiling, dense fumes; 420°C – Neck deposition, red base	407°C – Kajjali boiling, dense fumes; 420°C – Neck deposition, red base
30	412°C – Blue flame after Hot Shalaka	420°C – Dry red base, blue flame	420°C – Dry red base, blue flame

34	430°C – Yellow fumes, blue flame continues	425°C – Yellow fumes, red-hot base, blue flame	425°C – Yellow fumes, red-hot base, blue flame
36	—	425°C – Yellow fumes, blue flame	425°C – Yellow fumes, blue flame
38	420°C – Fumes markedly reduced, neck deposition increased	425°C – No fumes, no blue flame after Hot Shalaka	425°C – No fumes, no blue flame after Hot Shalaka
40	460°C – Orange-red base, copper coin test negative	475°C – Copper coin test positive, corking, Tivragni started	475°C – Copper coin test positive, corking, Tivragni started
44	450°C – Golden-red base, no further changes	575°C – Tivragni continued	575°C – Tivragni continued
46	479°C – Copper coin test positive, corking, Tivragni started	625°C – Tivragni continued	625°C – Heating stopped (EMF switched off)
48	540°C – Tivragni continued	640°C – Heating stopped	—
50	610°C – Tivragni continued	—	—
51	660°C – EMF stopped	—	—

Preparation of Shadgunabalijarita Rasasindoora:-





Pharmaceutical Evaluation:-

Critical observations were recorded throughout the Kupipakwa process to monitor the pharmaceutical preparation. These observations included colour changes, consistency, flame characteristics, evolution of fumes, corking stage, conclusion of the reaction and ultimate yield and % recovery. The prepared Shadgunabali jarita Rasa Sindoor was further assessed for its satisfactory preparation with classical Ayurvedic standards.

Pharmaceutico-Analytical Characterization:-

The prepared Shadgunabali jarita Rasa Sindoor was evaluated pharmaceutico-analytically for the establishment of quality and physicochemical features. Organoleptic features such as colour, odour, look and texture were noted. The physicochemical analysis was carried out according to the normal pharmacopoeial protocols. The constituent composition, crystalline phase and surface morphology were determined by instrumental characterisation using X-ray fluorescence (XRF), X-ray diffraction (XRD). All studies were performed under recognised laboratory methods(14).

In-vitro Cytotoxicity Assay:-

The cytotoxic effect of Shadgunabali jarita Rasa Sindoor was studied on human lung adenocarcinoma (A-549) cell line by MTT assay. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with foetal bovine serum and antibiotics under standard culture conditions (37°C, 5% CO₂). The test formulation was made at varied concentrations and incubated with A-549 cells in 96-well microplates for the indicated exposure time. Cell viability was assessed by MTT assay after treatment and the absorbance was detected using microplate reader. Cell viability was represented as a percentage of viable cells relative to the untreated control.(15)

Determination of IC₅₀:-

The percentage cell viability at varying concentration of test formulation was shown against dose response curves. The half-maximal inhibitory concentration (IC-50) was obtained using non-linear regression analysis.

Statistical Analysis:-

All experiments were performed in triplicate and findings are expressed as mean $\pm$ standard deviation (SD). Dose-response analysis and IC-50 determination were carried out using non-linear regression analysis. Statistical significance was accepted at p value <0.05

Results:-**Pharmaceutical Preparation of ShadgunabalijaritaRasa Sindoor:-****Table 1. Pharmaceutical Processing of Raw Materials**

Sr. No.	Pharmaceutical Process	Classical Reference	Initial Weight (g)	Final Weight (g)	Percentage Change (%)
1	Shodhana of Parada	Parada Samhita	108	105	-2.7
2	Shodhana of Gandhaka	Rasatarangini	614	602	-1.9
3	Preparation of Kajjali	Rasatarangini	700	675	-3.6
4	Bhavana of Kajjali	Rasatarangini	675	685	+1.4

Pharmaceutical Observations:-**Shodhana of Parada:-**

- The weight loss after purification was from 108 g to 105 g.
- Purified Parada had a better lustre and mobility.
- Visible foreign matter was removed.

Shodhana of Gandhaka:-

- Weight was reduced from 614 g to 602 g.
- Purified Gandhaka was bright yellow, supple and free from obvious contaminants.

Preparation of Kajjali:-

- Kajjali became a uniform coloured, fine, lusterless black powder.
- Classical quality checks showed Rekhapurnata and Nishchandrata acceptable.

Bhavana of Kajjali:-

- Weight increased from 675 g to 685 g due to uptake of the Bhavana medium.
- The fineness was increased and material was made more uniform.

Preparation of Shadgunabalijarita Rasa Sindoor:-

- Successfully prepared three batches.
- The time of heating was 48 to 51 hours.
- The final yield of Rasa Sindoor was in the range of 28 g to 30 g.
- The substance was a vivid red crystalline powder, as described in classical literature.

Table 2. Summary of Pharmaceutical Preparation of Shadgunabalijarita Rasa Sindoor

Parameter	Observation
Initial material	ShadgunabalijaritaKajjali
Pharmaceutical method	Classical KupipakwaRasayana
Heating schedule	Mridu → Madhyama → Tivraghi
Number of batches	3
Heating duration	48–51 h
Final appearance	Bright red crystalline powder
Texture	Fine and smooth
Lustre	Characteristic bright lustre
Final yield	28–30 g per 200 g Kajjali
Batch reproducibility	Satisfactory

Results:-

The conventional KupipakwaRasayanaprocess was successfully used to prepare ShadgunabalijaritaRasa Sindoor. Physicochemical changes were noticed sequentially during the pharmaceutical processing, suggesting the gradual transformation of Kajjali into the finished product. The original homogenous lustreless black Kajjali underwent characteristic changes during regulated heating. These changes included evolution of fumes, deposition of sublimed material in the neck of the Kacha Kupi and successful corking at the proper stage. The final product was obtained as a fine bright red crystalline powder deposited in the neck of the container after self-cooling, as in the ancient descriptions. The preparation was reproducible in all three batches with heating time of 48-51 h and had a constant yield of 28-30 g per 200 g Kajjali.

Table 3. Batch-wise Preparation of Shadgunabalijarita Rasa Sindoor Reference: Rasatarangini, Chapter 6, Verses 162–167

Batch	Quantity of Kajjali Taken (g)	Heating Duration (Hours)	Final Yield (g)	Yield %
I	200	51	28	14

II	200	48	30	15
III	200	48	30	15

Organoleptic Evaluation:-

The last Shadgunabali jarita Rasa Sindoor demonstrated organoleptic features that were indicative of adequate pharmaceutical processing. Formulation was brilliant red fine crystalline powder with smooth texture and free from apparent foreign materials. No evidence of any disagreeable smell or dampness was discovered indicating acceptable stability and suitable storage conditions.(16)

Table 2. Organoleptic Characteristics

Parameter	Observation
Colour	Bright red
Appearance	Fine crystalline powder
Texture	Smooth
Odour	Odourless / characteristic
Moisture	Absent
Foreign matter	Not detected

Classical Quality Assessment:-

The developed formulation was found to meet the conventional quality requirements prescribed for Rasa Sindoor. The pharmaceutical procedure was completed and the formulation was suitable for further analytical and biological evaluation, as confirmed by correct colour development, successful Kupipakwa transformation and excellent product recovery, which are characteristic pharmaceutical observations.

Physicochemical Evaluation:-

Physicochemical assessment of the produced formulation showed good quality features. The measured analytical values, low moisture content and acceptable inorganic residual, suggest satisfactory pharmaceutical processing and uniformity of the batch.(17)

Table 3. Physicochemical Parameters

Parameter	Observation
Loss on drying	0.51% W/W
Total ash	0.23% W/W
Acid insoluble ash	0.14% W/W
Water soluble extractive	0.11% W/W
pH	Not Recorded

Instrumental Characterization:-

Further instrumental characterisation confirmed the identification and structural features of the produced formulation. XRD showed the characteristic diffraction peaks of crystalline mercuric sulphide (α -HgS). XRF analysis verified mercury and sulphur as the major elemental components, supporting the predicted composition of Rasa Sindoor.

Table 4. Summary of Instrumental Analysis

Investigation	Major Findings
XRD	Characteristic peaks of mercuric sulphide
XRF	Predominantly mercury and sulphur, consistent with mercuric sulphide (HgS)

In-vitro Cytotoxic Activity Against A-549 Cell Line:-

Shadgunabali jarita Rasa Sindoor displayed concentration dependant cytotoxic activity against A-549 human lung cancer cell line. With increasing dosage of the formulation cell viability was gradually reduced demonstrating dose-dependent suppression of cancer cell proliferation. Microscopic studies showed morphological changes in treated cells including cell shrinkage and rounding, loss of cellular adherence and lower confluency compared to untreated control cells. These morphological changes also corroborated the cytotoxic effect of the mixture.

Table 5. Cytotoxic Activity Against A-549 Cell Line

Concentration	Cell Viability (%)	Absorbance average 3 batches (O.D)	Growth Inhibition (%)
Control	100	2.110	0
10	74.08	1.563	25.92

20	67.38	1.421	43.08
40	58.52	1.234	60.83
80	52.81	1.114	73.14
100	40.06	0.845	81.31

Effects of Sample – Shadgunabalijarit a Rasa Sindoor against A-549 (Human Lung Cancer Cell Line) by MTT Assay

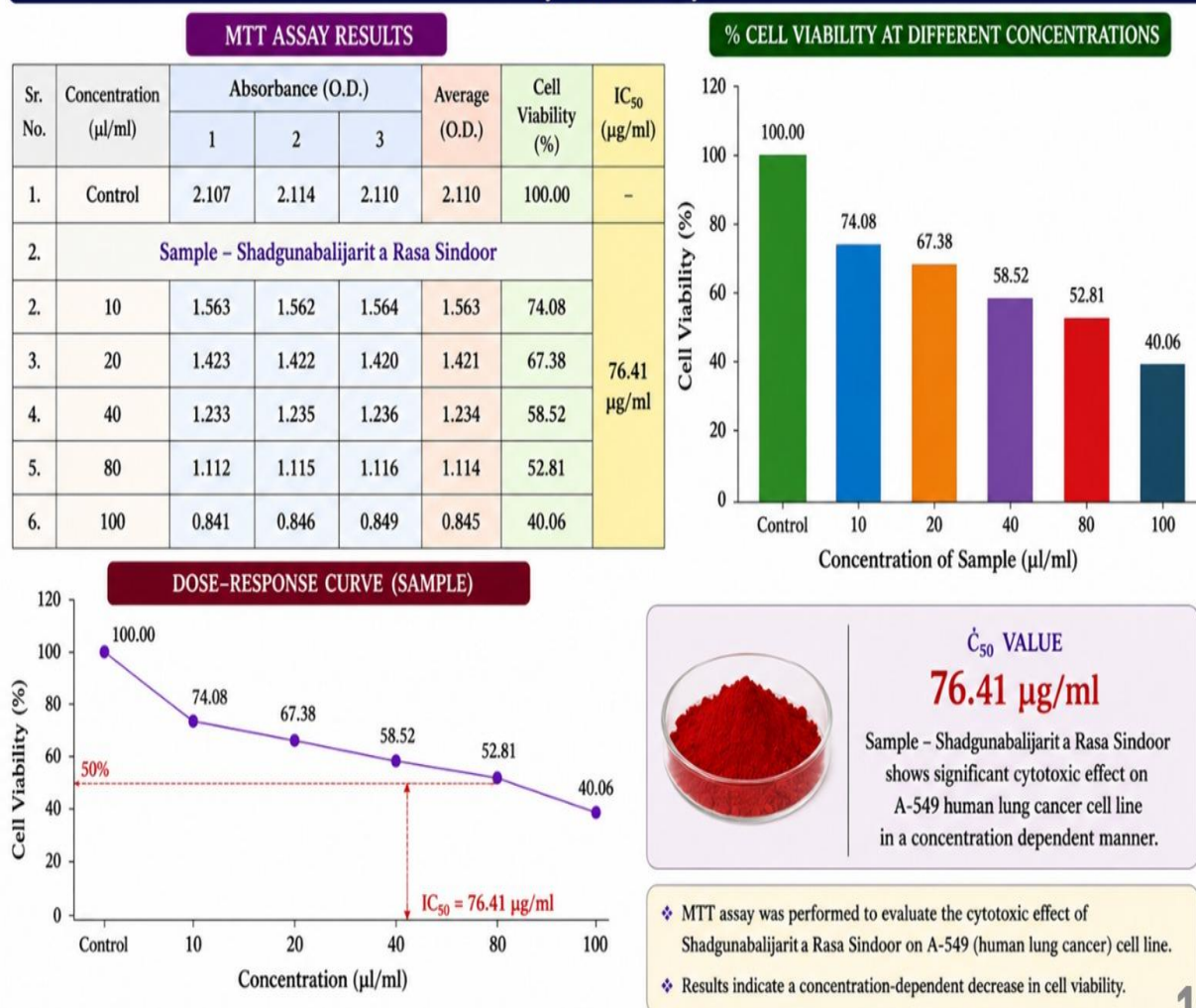


Figure 1. Dose-response curve showing concentration-dependent inhibition of A-549 cell viability by Shadgunabalijarita Rasa Sindoor.

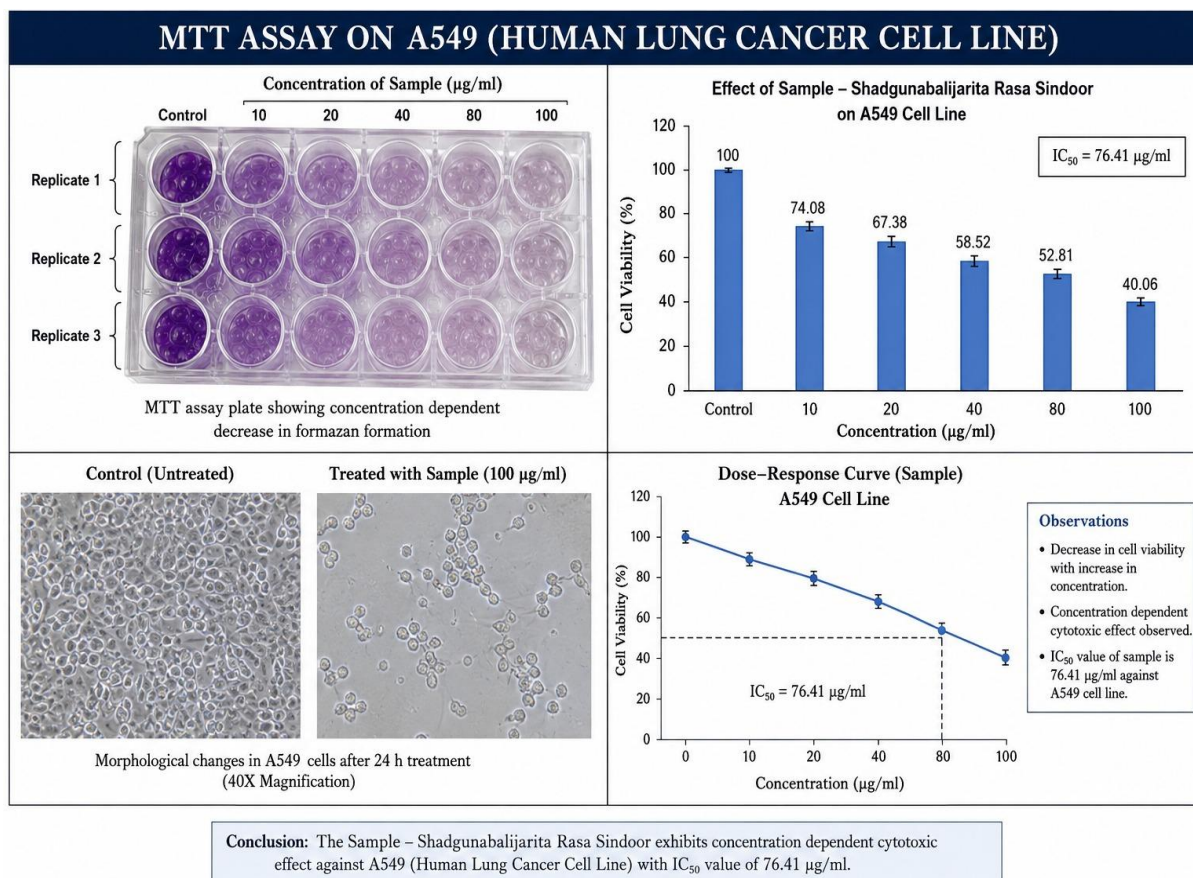


Figure 2. Representative microscopic images of untreated control and treated A-549 cells demonstrating concentration-dependent morphological alterations.

IC₅₀ Determination:-

Dose response research indicated measurable cytotoxic activity of ShadgunabaliaritaRasa Sindoor against A-549 cells. The IC₅₀ (the concentration that would inhibit 50% cell viability under the experimental conditions) was found to be 76.41 $\mu\text{g/mL}$. This finding verifies the in-vitro quantifiable antiproliferative effect of the formulation against A-549 cells.

Results Summary:-

The classical process of KupipakwaRasayanawas successfully adopted for the preparation of ShadgunabaliaritaRasa Sindoor and it presented characteristic organoleptic traits with good physicochemical quality. XRD showed the crystalline form of α -HgS and XRF showed the presence of mercury and sulphur as the main elemental components. The formulation exhibited cytotoxicity against A-549 cell line in a concentration dependent manner with an IC₅₀ of 76.41 $\mu\text{g/mL}$. This was further confirmed by the characteristic morphological alterations in the treated cells.

Discussion:-

Pharmaceutical Standardization of Shadgunabaliarita Rasa Sindoor:-

The successful preparation of ShadgunabaliaritaRasa Sindoor using classical KupipakwaRasayanatechniques shows that the pharmaceutical approach chosen was reproducible and suitable for generating the formulation with the characters stated in Rasashastra. Pharmaceutical observations during processing and consistent product recovery were good, distinctive bright red colour suggesting effective completion of the pharmaceutical transformation. Standardisation of purification, Kajjali preparation, controlled heating and endpoint determination gives a consistent platform for quality assurance and future batch repeatability.

Significance of XRD Analysis:-

XRD examination showed that the main crystalline phase in the final formulation was mercury sulphide (HgS) in the form of cinnabar (α -HgS). The diffraction pattern was in good agreement with the standard ICDD reference pattern (PDF 00-006-0256). No extra crystalline impurity peaks were found.

These results demonstrate that the Kupipakwa procedure successfully converted the initial materials to crystalline α -HgS rather than leaving visible crystalline mercury or sulphur. This gives objective scientific proof for the physicochemical transformations stated in classical Rasashastra. The lack of other crystalline phases also shows that the pharmaceutical process used resulted in a very uniform product with good phase purity, thereby supporting the reproducibility of the manufacturing process.

Significance of XRF Analysis:-

XRF elemental analysis showed that mercury and sulphur were the primary elemental constituents of the formulation with approximately 68.73 and 18.30%, respectively. These results are consistent with the expected composition of mercury sulphide produced during Kupipakwa processing. Some other elements were found also, albeit in trace amounts and apparently from mineral source materials or natural impurities. The detection of arsenic and lead by XRF should be used with caution as XRF is a quick elemental screening technology and does not give the analytical sensitivity or quantitative accuracy of ICP-MS or ICP-OES for toxicological evaluation. Hence, additional ICP-based investigation is still required before any judgements can be drawn on the safety of the heavy metals. The XRF results are in overall agreement with the effective synthesis of a sulphur-rich mercurial preparation commensurate with the expected medicinal content of conventional Rasa Sindoor.

Interpretation of Cytotoxic Activity:-

The present study found that the Shadgunabali jarita Rasa Sindoor had significant cytotoxic action on the human lung adenocarcinoma cell line, A-549. The number of viable cells progressively decreased with increasing concentration of the formulation, demonstrating a definite biological response that was dosage dependent. Cell viability was lowered from 74.08% at 10 μ g/mL to 40.06% at 100 μ g/mL, with an estimated IC_{50} value of 76.41 μ g/mL. These findings were also complemented by morphological inspection in which treated cells displayed cellular shrinkage, rounding, diminished adherence and disruption of normal monolayer architecture, all hallmark characteristics of cytotoxic damage. The formulation was less cytotoxic than the standard anticancer drug 5-fluorouracil (IC_{50} = 7.88 μ g/mL). However, the biological activity recorded still indicates that the formulation has appreciable antiproliferative potential that is worthy of further mechanistic investigation.

Correlation Between Classical Processing and Biological Activity:-

The present results are in general agreement with earlier pharmaceutico-analytical studies on Rasa Sindoor reporting crystalline α -HgS as the major phase after Kupipakwa processing. Similar studies have also confirmed mercury and sulphur as the major elemental ingredients therefore validating the repeatability of classical pharmacological procedures. But very few publications are available for in-vitro cytotoxic action of Shadgunabali jarita Rasa Sindoor. Thus the present inquiry is original.

Strengths of the Study:-

The present study involves ancient Ayurvedic drug manufacturing with modern analytical characterisation and biological evaluation. The Shadgunabali jarita Rasa Sindoor is fully characterised scientifically by standardised pharmaceutical processing, XRD phase identification, XRF elemental analysis and in-vitro cytotoxic assessment. Such comprehensive examination reinforces the scientific basis for future quality control and pharmaceutical investigations.

Limitations:-

The present study has certain limitations. Biological evaluation was restricted to an in-vitro model using a single human lung adenocarcinoma cell line, and the molecular mechanisms responsible for the observed cytotoxicity were not investigated. In addition, XRF provided elemental screening rather than definitive quantitative analysis of trace heavy metals. Therefore, ICP-based elemental analysis, mechanistic investigations, in-vivo studies and well-designed clinical studies are required before therapeutic conclusions can be drawn.

Future Perspectives:-

Future studies should investigate apoptosis, oxidative stress, mitochondrial dysfunction, cell-cycle arrest and molecular signalling pathways involved in the observed cytotoxic activity. Evaluation against additional cancer cell

lines, normal cell lines and suitable animal models would further clarify the therapeutic potential and safety profile of Shadgunabali jarita Rasa Sindoor.

Conflict of Interest:-

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgements:-

The authors express their sincere gratitude to the Department of Rasashastra and Bhaishajya Kalpana, Shri Ayurved College, Nagpur, for providing the necessary facilities, institutional support and academic guidance for conducting the present research. The authors also acknowledge the support extended during authentication of raw materials and express their gratitude to the analytical laboratory personnel for their valuable assistance in conducting physicochemical, microbiological and heavy metal analyses.

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