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REVIEWER'S REPORT

Manuscript No.: IJAR-58965

Title: Pharmaceutico-Analytical Standardization and In-vitro Anti-lung Cancer Activity of Shadgunabalijarita Rasa Sindoor Against A-549 Human Lung Adenocarcinoma Cell Line,

Recommendation:
Accept as it is .....
Accept after minor revision.....
Accept after major revision ...YES.....
Do not accept (*Reasons below*) .....

Rating	Excel.	Good	Fair	Poor
Originality		☑		
Techn. Quality			☑	
Clarity		☑		
Significance		☑		

Reviewer's ID: JPR- 094

Detailed Reviewer's Report

Below is a **reviewer-style report** suitable for an editorial assessment. I have treated the manuscript as a scientific paper and focused particularly on methodological rigor, analytical validity, cytotoxicity evidence, reproducibility, safety, and the distinction between *in-vitro cytotoxicity* and *anticancer activity*.

Reviewer Report

Manuscript Title

"Pharmaceutico-Analytical Standardization and In-vitro Anti-lung Cancer Activity of Shadgunabalijarita Rasa Sindoor Against A-549 Human Lung Adenocarcinoma Cell Line"

Overall Assessment

The manuscript addresses an interesting and potentially relevant topic at the intersection of **Rasashastra pharmaceutical standardization, modern analytical characterization, and in-vitro cancer biology**. The attempt to combine classical pharmaceutical processing with XRD, XRF and MTT-based cytotoxicity assessment is a potential strength.

However, in its present form, the manuscript has **substantial methodological, analytical, statistical, reporting, safety and interpretation problems**. The central biological conclusion—that the formulation possesses "anti-lung cancer activity"—is considerably stronger than what can

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be supported by the presented evidence. The study appears to demonstrate, at most, in-vitro cytotoxicity against one cancer cell line, and even that conclusion requires additional experimental details and controls.

There are also several internal inconsistencies in the manuscript, particularly concerning the experimental design, number of replicates, analytical characterization, XRF interpretation, MTT methodology, statistical analysis, and references.

Recommendation: Major Revision

If the authors cannot provide the underlying experimental data, raw analytical results, appropriate controls, and adequate methodological details, I would recommend rejection rather than major revision.

1. Strengths of the Manuscript

1.1 Relevant and interdisciplinary research question

The manuscript attempts to integrate:

- Classical Ayurvedic pharmaceutical preparation
- Pharmaceutical standardization
- XRD characterization
- XRF elemental analysis
- A549 lung cancer cell-line testing
- MTT cytotoxicity assay

This interdisciplinary approach could be valuable because it attempts to connect traditional pharmaceutical practices with contemporary analytical and biological methods.

1.2 Detailed description of the pharmaceutical process

The manuscript provides substantial information regarding:

- Shodhana of Parada
- Shodhana of Gandhaka
- Kajjali preparation
- Bhavana
- Jarana
- Kupipakwa Rasayana procedure

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- Heating schedule
- Temperature changes
- Corking
- Product deposition
- Yield

The three-batch preparation is particularly useful because batch-to-batch reproducibility is important for standardization.

1.3 Inclusion of multiple batches

The authors report three batches with:

- 200 g Kajjali per batch
- 48–51 h heating
- 28–30 g final product
- 14–15% yield

This is potentially a useful component of pharmaceutical standardization.

However, the manuscript needs to demonstrate statistically or analytically that the batches are actually equivalent rather than simply reporting similar yields.

1.4 Use of XRD

XRD is an appropriate technique for determining the crystalline phases present in a mercury-sulfur preparation.

The reported identification of α-HgS/cinnabar is scientifically relevant and potentially useful for establishing the solid-state composition of the final product.

1.5 Use of XRF

XRF can provide useful elemental screening and can establish that Hg and S are major elements.

The authors appropriately acknowledge that XRF has limitations for trace toxic-element quantification. This is one of the more scientifically responsible statements in the manuscript.

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1.6 Attempt to quantify biological activity

The manuscript does not merely state that the formulation was "active"; it provides:

- Concentration-dependent viability data
- Growth inhibition
- Absorbance values
- IC_{50}
- Morphological observations

The reported IC_{50} of 76.41 µg/mL gives a quantitative measure that can be compared with other studies.

1.7 Limitations are acknowledged

The authors recognize several limitations, including:

- Single cancer cell line
- Lack of mechanistic investigation
- Lack of in-vivo studies
- Need for ICP-based elemental analysis
- Need for additional safety studies

This is appropriate, although the manuscript's conclusions should be further restricted to reflect these limitations.

2. Major Weaknesses

2.1 The title overstates the evidence

The most important problem is the phrase:

"Anti-lung Cancer Activity"

The experiment uses only one cancer cell line, A549, and an MTT assay.

A decrease in metabolic activity in A549 cells does not establish anti-lung-cancer activity in the therapeutic sense.

The appropriate terminology would be:

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"in-vitro cytotoxicity against A549 human lung adenocarcinoma cells"

rather than:

"anti-lung cancer activity."

The authors should avoid implying therapeutic efficacy.

Suggested title

"Pharmaceutico-Analytical Standardization and In-vitro Cytotoxicity of Shadgunabalijarita Rasa Sindoor Against A549 Human Lung Adenocarcinoma Cells"

This would be considerably more scientifically defensible.

3. Major Methodological Concern: MTT Assay Is Insufficiently Described

The biological methodology is currently inadequate for reproducibility.

The manuscript states that cells were exposed to different concentrations, but it does not clearly report:

- Exact concentration units and preparation
- Number of cells/well
- Volume per well
- Exposure duration
- Number of independent experiments
- Number of technical replicates
- MTT concentration
- MTT incubation duration
- Solubilization reagent
- Wavelength used for absorbance
- Blank correction
- Vehicle control
- Positive control
- Preparation/sterilization of the formulation
- Whether formulation particles interfered with absorbance
- Whether the test material itself reduced MTT
- Whether precipitation occurred in wells

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These details are essential.

Particularly important

Because this is a colored mineral preparation, an MTT assay can potentially be affected by optical interference or interaction of the test material with the assay system.

The authors should demonstrate appropriate:

- Cell-free formulation blanks
- MTT/formulation interference controls
- Vehicle controls
- Positive controls

Without these controls, the reported IC_{50} cannot be considered sufficiently validated.

4. The Replication Statement Is Ambiguous

The manuscript says:

"All experiments were performed in triplicate"

but Table 5 states:

"Absorbance average 3 batches"

These are not necessarily equivalent.

The authors need to distinguish:

- **Biological replicates**
- **Technical replicates**
- **Independent experimental runs**
- **Three pharmaceutical batches**

For example, if each of the three pharmaceutical batches was independently tested three times, the design is different from simply testing one pooled sample in triplicate.

The authors should provide a clear experimental structure such as:

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Three independently prepared pharmaceutical batches were tested, with three independent biological experiments per batch and X technical wells per concentration.

At present, the reader cannot determine the true sample size.

5. The IC_{50} Calculation Needs Verification

The manuscript reports:

IC_{50} = 76.41 µg/mL

However, the concentration-response dataset is:

Concentration	Viability
10 µg/mL	74.08%
20 µg/mL	67.38%
40 µg/mL	58.52%
80 µg/mL	52.81%
100 µg/mL	40.06%

This deserves careful statistical examination.

The authors should provide:

- The actual dose-response curve
- Regression model used
- Equation or nonlinear model
- 95% confidence interval for IC_{50}
- Goodness-of-fit
- Individual replicate values
- Error bars
- Software used

Importantly, the data appear to cross approximately 50% viability between 80 and 100 µg/mL, so the reported IC_{50} may be plausible, but **the manuscript does not provide sufficient information to independently assess how 76.41 µg/mL was derived**.

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6. No Proper Statistical Comparison Is Presented

The manuscript states:

"Statistical significance was accepted at p value <0.05"

but no actual statistical test results are presented.

There are no:

- p-values
- confidence intervals
- test statistics
- ANOVA results
- post-hoc comparisons
- error bars in the table

Therefore, the statement regarding statistical significance is essentially unsupported.

The authors should specify the statistical test appropriate to the experimental design and present the results.

7. The 5-Fluorouracil Comparison Is Problematic

The Discussion states:

"The formulation was less cytotoxic than the standard anticancer drug 5-fluorouracil (IC50 = 7.88 µg/mL)."

But **5-fluorouracil does not appear adequately described in the Materials and Methods**, nor is it included in Table 5.

This creates a major problem.

If 5-FU was actually experimentally tested, the authors must provide:

- Concentrations tested
- Experimental conditions
- Replicate number

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- Viability data
- IC_{50} calculation
- Statistical comparison

If 7.88 µg/mL was obtained from a published source, the source must be clearly cited and the comparison should be described as a **literature comparison**, not as a direct experimental comparison.

8. XRF Results Are Incomplete

The Discussion reports:

Hg = 68.73%
S = 18.30%

but the Results table merely says:

"Predominantly mercury and sulphur."

This is inconsistent.

If quantitative XRF data are available, the complete elemental composition should be presented, including:

- Hg
- S
- Pb
- As
- Other detected elements
- Analytical units
- Detection limits, if available
- Replicates
- Measurement uncertainty

The authors also mention arsenic and lead but do not provide their actual concentrations.

This is particularly important because the formulation contains **mercury**.

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9. Safety Issue Requires Much Greater Attention

This is arguably the most important concern from a translational perspective.

The manuscript describes a preparation containing crystalline HgS, while also claiming anticancer potential.

Demonstrating cytotoxicity against A549 cells is not sufficient to demonstrate selective anticancer action.

The study needs to address:

Is the formulation selectively toxic to cancer cells, or is it simply toxic to cells because of its mercury content?

This question is currently unanswered.

At minimum, future or revised work should consider:

- Normal human lung cells
- Appropriate non-malignant cell line
- Cancer-selectivity index
- Detailed elemental analysis
- Heavy-metal quantification
- Toxicity assessment

The current study cannot support a claim that the formulation is a safer anticancer agent.

10. XRD Interpretation Is Too Strong

The manuscript states:

"No extra crystalline impurity peaks were found."

and subsequently concludes that the material has:

"good phase purity"

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This is too strong based solely on XRD.

XRD can identify **crystalline phases**, but it cannot establish that there are no amorphous components or that all impurities are absent.

Similarly, the absence of detectable diffraction peaks does not prove the complete absence of an element or impurity.

The authors should use language such as:

"No additional crystalline phases were detected within the detection limits of the XRD analysis."

This is scientifically more appropriate.

11. XRF Cannot Establish Chemical Speciation

The manuscript correctly recognizes some limitations of XRF, but the discussion still needs refinement.

XRF demonstrates elemental composition; it does not by itself establish that mercury is present specifically as HgS.

The conclusion that Hg and S constitute HgS should primarily be supported by **XRD/other appropriate chemical-state or spectroscopic evidence**, not XRF alone.

12. Pharmaceutical Standardization Is Not Yet Fully Established

The manuscript repeatedly uses the term **"standardization."**

However, genuine pharmaceutical standardization generally requires a much more comprehensive set of quality attributes.

The study currently provides:

- Organoleptic assessment
- Basic physicochemical parameters

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- XRD
- XRF

But several potentially important parameters are absent.

For example:

- Particle-size distribution
- Morphology by SEM
- FTIR/Raman characterization
- Quantitative Hg/S analysis by ICP-OES or ICP-MS
- Trace-element analysis
- Microbial limits
- Stability studies
- Batch-to-batch analytical comparison
- Moisture/water activity where relevant
- Appropriate validated analytical methods

Therefore, the manuscript may be better described as **"pharmaceutico-analytical characterization"** rather than claiming comprehensive standardization.

13. Internal Inconsistencies in Tables and Numbering

There are several editorial and scientific inconsistencies.

For example:

- Table 1 appears in multiple places with different data.
- Table 2 is used for pharmaceutical heating observations and later again for organoleptic characteristics.
- Table 3 is used for both batch preparation and physicochemical parameters.
- "Results" appears more than once.
- "3. Results Summary" is followed by "4. Discussion," but the structure is inconsistent.
- "Preparation of Shadgunabalijarita Rasasindoora" is incomplete in the supplied text.
- Figures 1 and 2 are mentioned, but their actual content is absent from the manuscript supplied for review.

The manuscript needs complete restructuring and renumbering.

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14. Reference 16 Is Not an Appropriate Reference

Reference 16 appears to be:

"The last Shadgunabalijarita Rasa Sindoor demonstrated organoleptic features…"

This is not a bibliographic reference. It appears to be text generated from the manuscript itself.

It should be removed and replaced with the actual source supporting the relevant analytical/pharmaceutical statement.

This is a significant reference-quality problem.

15. References 3, 4, 5, 10 and 12 Are Repetitive or Poorly Assigned

The manuscript repeatedly cites *Rasatarangini* but does not consistently identify:

- Chapter
- Verse
- Edition
- Page number

For classical Ayurvedic pharmaceutical procedures, precise textual citation is especially important.

For example, the exact verse used for:

- Shadguna Jarana
- Bhavana
- Kupipakwa procedure
- Heating/end point

should be explicitly provided.

16. Some Claims Are Not Supported by the Data

Examples include:

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"good pharmaceutical properties"

"excellent product recovery"

"very uniform product with good phase purity"

"appreciable antiproliferative potential"

"scientific proof"

These statements should be moderated.

The study provides preliminary experimental evidence, not proof of therapeutic efficacy.

17. "Pharmacological" vs "Pharmaceutico-Analytical"

The manuscript sometimes uses terms such as:

"pharmacological changes"

for pharmaceutical processing.

This terminology should be corrected.

The observed changes during heating are primarily physicochemical/pharmaceutical transformations, unless pharmacological activity has specifically been demonstrated.

18. No Evidence of Selectivity

This is a major biological weakness.

The authors tested A549 cells but apparently did not test:

- Normal lung cells
- Normal fibroblasts
- Other normal human cells
- Another cancer cell line

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Consequently, there is no evidence that the formulation preferentially affects cancer cells.

A formulation that kills A549 cells is not necessarily an anticancer drug.

19. No Mechanistic Evidence

The authors themselves acknowledge this limitation.

There is no evidence concerning:

- Apoptosis
- Necrosis
- Caspase activation
- ROS
- Mitochondrial membrane potential
- Cell-cycle arrest
- DNA damage
- Molecular signaling

Therefore, the biological conclusions should remain preliminary.

20. The Manuscript Should Address Potential MTT Interference

This deserves explicit emphasis.

Rasa Sindoor is a strongly colored inorganic/mineral preparation.

The authors should determine whether the material itself contributes to absorbance at the measurement wavelength or interacts chemically with MTT.

Appropriate **cell-free controls containing the formulation plus MTT** are essential.

Without this, an apparent reduction in MTT signal could theoretically arise from assay interference rather than reduced cellular viability.

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21. Key Scientific Question: Is the Observed Effect Due to HgS?

The manuscript identifies α-HgS as the major crystalline phase.

Therefore, an important unanswered question is:

Is the observed cytotoxicity attributable to HgS, another component, residual impurities, released mercury species, or nonspecific toxicity?

The current design cannot answer this.

A stronger study would compare:

- Shadgunabalijarita Rasa Sindoor
- Appropriate HgS reference/control
- Vehicle
- Positive anticancer control
- Normal cells

and potentially investigate mercury/speciation under relevant biological conditions.

22. Novelty Needs to Be Reconsidered

The manuscript claims:

"the present inquiry is original."

This statement should not be made without a comprehensive literature review.

Moreover, because there appears to be a **previously published protocol concerning Shadgunabalijarita Rasa Sindoor and A549 cells**, the authors need to clearly distinguish the present completed experimental study from any previously published protocol or related publication.

The manuscript should explicitly state:

- What was previously published?
- What is new in the present study?

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- Whether the current manuscript is the subsequent completed study corresponding to a previously published protocol
- Whether any data have appeared previously

This is particularly important for publication ethics and transparency.

23. Minor/Editorial Issues

There are extensive language and formatting problems.

Examples:

- "Anti-lung Cancer" should be reconsidered.
- "A-549" should preferably be standardized as **A549**.
- "concentration dependant" → **concentration-dependent**
- "IC-50" → **IC_{50}**
- "pharmacutico" → **pharmaceutico**
- "because to late diagnosis" → grammatically incorrect
- Excessive repetition of the same findings
- Inconsistent capitalization
- Missing spaces between words
- Inconsistent use of "Rasa Sindoor," "Rasasindoor," and "Rasasindura"
- Inconsistent table numbering
- Incomplete figure information
- Several sentences appear machine-translated or poorly edited

A thorough language revision is required.

24. Key Points of the Manuscript

The principal scientific contributions can be summarized as:

1. **Classical pharmaceutical preparation** of Shadgunabalijarita Rasa Sindoor was attempted using Kupipakwa Rasayana methodology.
2. **Three batches** were prepared and showed broadly similar heating duration and yield.
3. The product was reported as a **bright red crystalline material**.
4. **XRD indicated α-HgS/cinnabar** as the principal crystalline phase.
5. **XRF indicated Hg and S as major elements.**
6. A549 cells demonstrated **concentration-dependent reduction in MTT viability**.
7. A reported **IC_{50} of 76.41 µg/mL** was obtained.
8. The study provides preliminary evidence for **in-vitro cytotoxicity**, but not therapeutic anticancer efficacy.

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25. Significance of the Study

The study has potential significance because it attempts to establish a bridge between traditional Rasashastra pharmaceutical processing and contemporary analytical/biological evaluation.

If properly validated, the work could contribute to:

- Documentation of reproducible preparation procedures
- Understanding of the physicochemical nature of Rasa Sindoor
- Development of quality-control parameters
- Generation of preliminary biological data
- Identification of areas for mechanistic and toxicological research

However, the clinical significance is currently very limited. The work should be positioned as an early-stage in-vitro investigation, not evidence of an effective lung-cancer treatment.

26. Essential Revisions Required Before Publication

I would require the authors to address the following points before reconsideration:

Essential scientific revisions

1. **Change the title** from "anti-lung cancer activity" to "in-vitro cytotoxicity."
2. Provide the **complete MTT experimental protocol**.
3. Clarify biological vs technical replicates.
4. Provide **raw/individual replicate data**.
5. Provide error bars and appropriate statistical analysis.
6. Provide the complete **dose-response curve and IC_{50} confidence interval**.
7. Explain precisely how IC_{50} = 76.41 μg/mL was calculated.
8. Provide appropriate **vehicle, positive, blank and assay-interference controls**.
9. Clarify whether 5-FU was experimentally tested or cited from literature.
10. Provide complete XRD diffraction data/figure and analytical conditions.
11. Provide complete XRF elemental results, including Pb and As.
12. Perform or provide **ICP-MS/ICP-OES or another validated quantitative elemental analysis**, especially for Hg, Pb and As.
13. Provide evidence of **batch-to-batch analytical reproducibility**, not merely similar yield.
14. Add normal/non-cancerous cell-line testing if claiming anticancer selectivity.
15. Moderate claims concerning safety and therapeutic efficacy.
16. Explain the potential biological significance of HgS-related cytotoxicity.
17. Correct all references and remove the invalid Reference 16.

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18. Clarify the relationship between this manuscript and any previously published protocol/study on the same formulation and A549 model.
19. Provide the actual figures and supplementary/raw analytical data.
20. Conduct comprehensive language and structural editing.

27. Reviewer Recommendation

Recommendation: MAJOR REVISION

Rationale: The subject is potentially interesting and the combination of classical pharmaceutical processing with XRD, XRF and cell-based testing has scientific value. The pharmaceutical preparation and preliminary characterization could constitute a useful contribution.

However, the current manuscript does not yet provide sufficient methodological detail or experimental evidence to support its stronger claims of anticancer activity or comprehensive standardization. The MTT methodology, replication structure, statistical analysis, assay controls, elemental safety characterization and interpretation of the cytotoxicity results require substantial improvement.

The reported IC_{50} of 76.41 µg/mL should not be presented as evidence of therapeutic anti-lung-cancer activity without additional selectivity, mechanistic and safety data.

If the authors can provide the underlying raw experimental data and adequately address the above methodological concerns, the manuscript could become publishable after major revision. If these data cannot be verified or supplied, rejection would be appropriate.

Suggested editorial decision

Major Revision — Reconsider after substantial methodological and data-quality revision.

Yes. Major Revision is justified because the manuscript has a potentially interesting pharmaceutico-analytical/cytotoxicity study, but there are substantial problems in methodological completeness, data consistency, statistical reporting, analytical validation, safety interpretation, controls, and overstatement of conclusions. Some issues are serious enough that the results cannot currently be independently reproduced or confidently interpreted.

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Below is a line-by-line reviewer-style justification that you can directly use in a peer-review report.

Reviewer Recommendation: Major Revision

Overall assessment

The manuscript addresses an interesting and potentially relevant topic by combining classical Rasashastra pharmaceutical processing with modern analytical characterization and an in-vitro cytotoxicity assay against A-549 cells. The integration of pharmaceutical standardization, XRD/XRF characterization and biological evaluation is a strength.

However, the manuscript in its current form requires major revision before it can be considered for publication. The principal concerns are not merely grammatical or editorial; they affect the scientific validity, reproducibility, interpretation and safety implications of the study. Important experimental details are missing, several numerical/data inconsistencies are present, the statistical treatment is inadequate, appropriate biological controls are insufficiently described, and some conclusions extend beyond what the presented data can establish.

Major concerns with line-by-line justification

1. Title: Lines 1–3

Current title:
"Pharmaceutico-Analytical Standardization and In-vitro Anti-lung Cancer Activity…"

Reviewer concern

The expression "anti-lung cancer activity" is too strong for a study performed only on one cancer cell line using an MTT assay.

The study demonstrates in-vitro cytotoxicity against A-549 cells, not anti-lung-cancer efficacy. A-549 cells are a cancer cell model and reduction in MTT signal does not establish anticancer efficacy in an organism.

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Required revision

Change the title toward:

"Pharmaceutico-Analytical Characterization and In-vitro Cytotoxicity of Shadgunabalijarita Rasa Sindoor against A-549 Human Lung Adenocarcinoma Cells"

This would accurately reflect the experimental evidence.

ABSTRACT

2. Lines 7–12 – Background

Concern

The statement that limitations of conventional therapy have resulted in interest in traditional formulations is broadly acceptable, but the manuscript does not sufficiently establish why Shadgunabalijarita Rasa Sindoor specifically warrants investigation.

The pharmacological claims are also inadequately supported.

Reviewer request

The authors should briefly explain:

- the classical indication(s) of the formulation;
- why this particular formulation was selected for lung cancer research;
- what previous evidence exists regarding its biological activity;
- what specific knowledge gap the study addresses.

The statement that evidence is "scarce" should be supported by an appropriate literature search/reference.

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3. Lines 14–18 – Objectives

Concern

The objectives are repetitive and partly vague.

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"Pharmaceutico-analytical features" is broad and does not specify the actual analytical endpoints.

Required revision

Clearly state the primary and secondary objectives, for example:

- preparation and batch-wise pharmaceutical standardization;
- physicochemical characterization;
- phase identification by XRD;
- elemental characterization by XRF;
- evaluation of cytotoxicity in A-549 cells;
- determination of IC50.

4. Lines 20–24 – Materials and Methods

Major concern

The phrase:

"after due purification and pharmaceutical processing"

is insufficiently detailed for reproducibility.

The manuscript should provide exact details of:

- purification procedures;
- quantities of reagents;
- duration of processing;
- number of triturations;
- Bhavana liquid quantity;
- duration of each Bhavana;
- Jarana procedure;
- heating rate;
- furnace type;
- temperature-control procedure;
- atmospheric conditions;
- cooling procedure.

Reviewer recommendation

The authors should provide sufficient procedural detail to allow another Rasashastra laboratory to reproduce the formulation.

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INTRODUCTION

5. Lines 41–47

Concern

The introduction states that conventional treatment has limitations and that safer agents are required. However, because the formulation contains mercury, the wording "safer" is particularly problematic.

The manuscript later detects mercury as the major constituent and also mentions arsenic and lead.

Major scientific issue

The authors should not imply that the investigated formulation is safer than conventional therapy without toxicological evidence.

Required change

Use neutral language such as:

"These limitations have encouraged investigation of additional therapeutic candidates, including compounds and formulations derived from traditional medicinal systems."

6. Lines 48–56

Concern

The statement that many anticancer drugs are of plant origin is not directly relevant to a mercury-containing herbo-mineral/mineral formulation.

The introduction moves from natural products to Ayurveda without adequately establishing the scientific rationale for a mercury-sulfur preparation.

Reviewer request

Strengthen the rationale specifically for Rasa Sindoor and HgS-containing formulations, including relevant analytical and toxicological literature.

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7. Lines 57–65

Major concern

The manuscript says:

"Such pharmacological changes have been credited with improved therapeutic capabilities…"

This is a potentially unsubstantiated therapeutic claim.

The authors need to distinguish:

- traditional/classical claims;
- physicochemical transformation;
- experimentally demonstrated biological effects.

A pharmaceutical transformation into α-HgS does not automatically establish improved therapeutic efficacy.

Required revision

The authors should explicitly state that such claims are derived from traditional literature unless supported by modern experimental evidence.

8. Lines 66–72 – Research gap

Positive point

This is one of the stronger sections because the manuscript identifies a potential research gap.

However

The claim that the study creates data supporting "anticancer potential" should be moderated because only one cell line was investigated.

A better formulation would be:

"The study provides preliminary in-vitro evidence of cytotoxic activity against A-549 cells."

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MATERIALS AND METHODS

9. Lines 92–98 – Procurement and authentication

Major concern

"Recognised sources" is not sufficient scientific documentation.

The authors should identify:

- supplier/manufacturer;
- batch/lot number;
- certificate of analysis, if available;
- purity;
- authentication method;
- date of procurement.

For mercury and sulfur, especially, source and purity are critical.

10. Lines 93–95 – Raw material characterization

Major concern

The authors state that mercury and sulfur were "tested for organoleptic characters."

This is insufficient for a formulation containing potentially toxic elements.

Required

Raw materials should ideally undergo analytical characterization for elemental impurities and purity.

At minimum, the manuscript should clearly state what identity and purity testing was performed.

2.3 PHARMACEUTICAL PREPARATION

11. Lines 101–115

Major concern: insufficient reproducibility

The formulation procedure is not sufficiently described.

For example:

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"Shadgunabalijarita Kajjali was prepared by doing later Jarana with sulphur…"

This sentence is unclear and grammatically problematic.

The exact Shadguna Bala/Jarana ratio is essential and should be reported explicitly.

Required

The authors should specify:

- amount of purified Parada;
- amount of purified Gandhaka;
- exact sulfur added during Shadguna Jarana;
- ratio of ingredients;
- number and duration of Jarana cycles;
- quantity and source of Kumari Swarasa;
- number of Bhavana cycles;
- duration of each cycle;
- drying conditions.

12. Lines 109–115 – Kacha Kupi and heating

Major concern

The manuscript says:

"seven layered Kacha Kupi"

but does not adequately describe the bottle preparation.

The authors should report:

- type of glass bottle;
- dimensions/capacity;
- number and thickness of cloth/clay layers;
- drying conditions;
- quantity filled into each bottle;
- furnace dimensions;
- furnace calibration;
- thermocouple position.

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13. Lines 109–110 – "Valuka Yantra/EMF"

Major concern

"Valuka Yantra/EMF" is unclear.

Are these two different heating systems or were different systems used in different batches?

If Batch I was prepared in one apparatus and Batches II–III in another, this could substantially affect batch comparability.

Required clarification

The authors should clearly define:

- EMF;
- furnace model;
- whether all batches used the same furnace;
- whether temperature was measured identically;
- whether heating programs were identical.

TABLE 1 / RAW MATERIAL WEIGHTS

14. Lines 105 and 167 – inconsistent data

This is a significant problem.

The first table reports:

Kajjali: initial weight —, final 675 g

while the later table reports:

Kajjali: 700 g → 675 g

This creates a data inconsistency.

Similarly, the tables are numbered incorrectly and repeatedly reuse "Table 1," "Table 2," and "Table 3."

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Required revision

All tables must be renumbered and cross-checked.

The authors must explain:

- whether Kajjali began at 700 g;
- whether 675 g was obtained before Bhavana;
- whether the same batch was used;
- how the 200-g aliquots used for final preparation relate to the 675/685-g material.

TABLE 2 – HEATING DATA

15. Lines 123–125

Major concern: batch variability

The heating profiles show substantial differences.

For example:

- Batch I reaches 660°C at 51 h.
- Batch II reaches 640°C at 48 h.
- Batch III reaches 625°C at 46 h.

Yet the manuscript concludes:

"Batch reproducibility satisfactory."

This conclusion is not adequately demonstrated.

Required

The authors should calculate and report:

- mean ± SD of heating duration;
- maximum temperature;
- yield;
- percentage recovery;
- between-batch variability.

The authors should explain why different endpoint temperatures and heating durations were considered equivalent.

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16. Heating temperatures

Major scientific concern

The temperature profile rises to approximately 625–660°C. Because mercury-containing material is being processed, the manuscript should provide a detailed discussion of:

- containment;
- fume management;
- laboratory occupational safety;
- mercury vapor control;
- furnace exhaust;
- personnel protection.

This is especially important given the substantial mercury content later reported by XRF.

17. Lines 111–114 – observations

Concern

The manuscript reports qualitative observations such as:

- blue flame;
- yellow fumes;
- sulfur smell;
- deposition.

However, these observations are not systematically documented.

Required

Provide photographs or a structured observation table, where appropriate, and clearly distinguish observed findings from interpretations.

PHARMACEUTICAL EVALUATION

18. Lines 128–134

Major concern

The authors say the product was assessed according to "classical Ayurvedic standards," but the actual acceptance criteria are not specified.

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Terms such as:

- Rekhapurnata;
- Nishchandratva;
- appropriate red color;
- classical endpoint

need operational definitions and references.

Required

Explain exactly:

- who performed the classical tests;
- how they were performed;
- what constituted a pass/fail result;
- whether the tests were performed independently by more than one assessor.

ANALYTICAL CHARACTERIZATION

19. Lines 136–144

Major methodological deficiency

The manuscript states that XRD and XRF were performed but gives almost no experimental details.

For XRD, report:

- instrument manufacturer/model;
- radiation source;
- wavelength;
- scanning range;
- step size;
- scan rate;
- voltage/current;
- sample preparation;
- database/software used;
- phase-identification method.

For XRF, report:

- instrument/model;
- calibration;
- sample preparation;

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- analytical mode;
- detection limits;
- units;
- uncertainty;
- reference standards.

Without these details, the analytical results are not reproducible.

PHYSICOCHEMICAL PARAMETERS

20. Lines 229–235

Major concern

The table reports:

- Loss on drying = 0.51%
- Total ash = 0.23%
- Acid insoluble ash = 0.14%
- Water soluble extractive = 0.11%
- pH = Not Recorded

The omission of pH is not necessarily fatal, but the authors should explain why it was not determined if it is part of the intended physicochemical characterization.

More importantly, the methods for each parameter are not described.

Required

Specify:

- analytical method;
- sample quantity;
- temperature;
- duration;
- number of replicates;
- calculation method;
- reference pharmacopoeial method.

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XRD RESULTS

21. Lines 236–243

Major concern

The authors claim:

"XRD showed the characteristic diffraction peaks of crystalline mercuric sulphide (α-HgS)."

However, the manuscript does not provide the actual diffraction pattern or peak positions.

Required

The manuscript should provide:

- XRD diffractogram;
- major 2θ values;
- corresponding d-spacings;
- reference database card;
- peak matching;
- preferably phase quantification or at least a clear statement that the analysis was qualitative.

22. Lines 309–320

Major overinterpretation

The statement:

"No extra crystalline impurity peaks were found."

does not prove that no impurities are present.

XRD primarily detects crystalline phases and may not detect:

- amorphous phases;
- low-concentration phases;
- poorly crystalline materials;
- phases below detection limits.

Therefore:

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"The lack of other crystalline phases also shows … very uniform product with good phase purity"

is too strong.

Required revision

Use:

"No additional crystalline phases detectable under the analytical conditions were identified."

Do not equate this with complete chemical purity or safety.

XRF RESULTS

23. Lines 322–336

Major concern

The manuscript reports approximately:

- Hg = 68.73%
- S = 18.30%

but does not provide the complete elemental composition.

If arsenic and lead were detected, their concentrations must be reported quantitatively if the instrument permits, or clearly reported as detected/not quantified.

Critical issue

The statement that XRF is insufficient for toxicological evaluation is correct, but this creates a major unresolved safety issue.

If the formulation contains detectable Pb and As, the authors cannot make broad statements about "good quality" without quantitative impurity assessment.

Required

Perform or provide validated:

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- ICP-MS;
- ICP-OES;
- AAS, where appropriate,

for Hg, Pb, As and other relevant toxic elements.

CYTOTOXICITY METHODS

24. Lines 145–154

Major methodological deficiency

The MTT assay is inadequately described.

Critical information is missing:

- A-549 cell source;
- authentication;
- passage number;
- mycoplasma testing;
- seeding density;
- volume/well;
- treatment duration;
- concentration preparation;
- solvent/vehicle;
- vehicle concentration;
- number of technical replicates;
- number of independent biological experiments;
- MTT concentration;
- incubation time;
- wavelength;
- blank correction;
- positive control.

These are necessary for reproducibility.

25. Missing vehicle and formulation controls

Major concern

This is particularly important for a particulate/mineral formulation.

The formulation may interfere with MTT through:

- optical absorbance;
- particle scattering;

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- chemical reduction of MTT;
- precipitation;
- direct interaction with assay reagents.

Required controls

The study should include:

- untreated cells;
- vehicle control;
- formulation without cells + MTT;
- formulation without MTT where appropriate;
- positive cytotoxic control;
- preferably a standard anticancer drug under the same experimental conditions.

Without these controls, reduced MTT signal cannot unequivocally be attributed to reduced cell viability.

26. Lines 156–164 – IC50 and statistics

Major concern

The manuscript states:

"All experiments were performed in triplicate"

but this is ambiguous.

Does "triplicate" mean:

- three wells from one experiment, or
- three independent biological experiments?

These are not equivalent.

Required clarification

State:

"Three independent experiments were performed, with X technical replicates per concentration in each experiment."

If only three wells were measured once, the statistical inference is weak.

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27. Statistical analysis is incomplete

The manuscript reports an IC50 but does not provide:

- 95% confidence interval;
- goodness of fit;
- regression parameters;
- statistical comparison with controls;
- p-values for individual concentrations;
- multiple-comparison correction where relevant.

Required

Provide the dose-response model, fitting method and IC50 with 95% CI.

RESULTS – CYTOTOXICITY

28. Lines 244–252

Major concern

The authors repeatedly describe the effect as:

"cytotoxicity"

but also call it:

"suppression of cancer cell proliferation."

An MTT assay measures metabolic activity, not necessarily proliferation or cell death specifically.

Required wording

Use:

"reduction in metabolic activity/cell viability as measured by the MTT assay."

Do not claim apoptosis, proliferation inhibition or a particular mechanism without additional experiments.

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29. Table 5 contains a major numerical inconsistency

The table gives:

Concentration	Cell viability	Growth inhibition
10	74.08%	25.92%
20	67.38%	43.08%
40	58.52%	60.83%
80	52.81%	73.14%
100	40.06%	81.31%

These values are internally inconsistent.

For example, if viability is 74.08%, inhibition calculated as:

100 − 74.08 = 25.92%

which is correct.

But for 20 µg/mL:

100 − 67.38 = 32.62%, not 43.08%.

For 40 µg/mL:

100 − 58.52 = 41.48%, not 60.83%.

For 80 µg/mL:

100 − 52.81 = 47.19%, not 73.14%.

For 100 µg/mL:

100 − 40.06 = 59.94%, not 81.31%.

This is a major data-quality problem.

The authors must verify the original raw data and correct the table.

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30. Absorbance versus viability inconsistency

If control OD = 2.110 and the 10 µg/mL OD = 1.563:

1.563 / 2.110 × 100 ≈ **74.1%**

which agrees with the reported 74.08%.

However, at 20 µg/mL:

1.421 / 2.110 × 100 ≈ **67.35%**, again matching the reported viability.

Therefore the **viability column appears to be calculated from OD**, whereas the "growth inhibition" column appears to have been calculated by another method or incorrectly entered.

This needs complete verification.

31. IC50 value requires verification

The reported IC50 is **76.41 µg/mL**.

Given the reported viability data, this may be plausible, but because the growth-inhibition column is inconsistent and the number of experimental replicates is unclear, the IC50 calculation must be independently checked.

Required

Provide:

- dose-response graph;
- raw replicate values;
- fitted curve;
- equation/model;
- IC50;
- 95% CI;
- R^2 or appropriate goodness-of-fit information.

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32. Lines 278–280 – microscopy

Concern

The manuscript says that morphological changes were observed, but the methodology is inadequate.

Missing:

- microscope type;
- magnification;
- imaging conditions;
- number of fields;
- method of image selection;
- whether images were representative or quantified.

Major issue

Morphological changes alone cannot establish the mechanism of cell death.

DISCUSSION

33. Lines 300–308

Concern

The authors conclude that the process was "reproducible" based on only three batches and similar yield.

Three batches can provide preliminary evidence of process consistency, but not comprehensive manufacturing reproducibility.

Required wording

Use:

"The three experimental batches showed preliminary batch-to-batch consistency…"

rather than claiming full reproducibility.

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34. Lines 309–320 – XRD interpretation

Major concern

The manuscript states:

"This gives objective scientific proof…"

This is too strong.

XRD supports phase identification, but does not establish:

- therapeutic efficacy;
- safety;
- complete conversion;
- absence of all impurities;
- pharmacological superiority.

Required revision

Replace "scientific proof" with:

"provides analytical evidence supporting the identification of crystalline α-HgS in the final product."

35. Lines 322–336 – safety issue

The manuscript correctly acknowledges the limitations of XRF, but then concludes:

"effective synthesis…"

and describes the preparation as having "medicinal content."

Major concern

Detection of Hg, Pb and As requires much stronger safety discussion.

The manuscript should not imply that formation of α-HgS makes the formulation clinically safe.

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Chemical form can influence bioavailability and toxicity, but safety requires experimental toxicokinetic/toxicological evidence.

36. Lines 337–353 – "significant cytotoxic action"

Major concern

The phrase:

"significant cytotoxic action"

is statistically inappropriate unless a statistical test demonstrates significance.

A dose-response trend does not automatically establish statistical significance.

Required

Either provide statistical comparisons and p-values or use:

"concentration-dependent reduction in A-549 cell metabolic activity."

37. Comparison with 5-fluorouracil

The manuscript states:

"5-fluorouracil (IC50 = 7.88 µg/mL)."

Major concern

It is unclear whether 5-FU was actually tested within this experiment or whether this number was taken from another publication.

If it was not tested under identical conditions, direct comparison is scientifically inappropriate.

Required

If 5-FU was tested:

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- describe its concentration range;
- treatment duration;
- source;
- replicate number;
- statistical comparison.

If the value came from literature, state explicitly that it is a literature-derived value and should not be directly compared quantitatively.

38. Lines 355–362 – novelty claim

Concern

The statement:

"the present inquiry is original"

requires evidence.

The authors should perform a systematic or at least comprehensive literature search and explain what previous studies have and have not reported.

Required

Avoid an absolute novelty claim unless supported by a documented literature search.

Use:

"To the best of the authors' knowledge, limited published evidence is available specifically regarding the in-vitro cytotoxicity of Shadgunabalijarita Rasa Sindoor against A-549 cells."

39. Lines 363–370 – "fully characterised scientifically"

Major concern

The manuscript says the formulation is:

"fully characterised scientifically"

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This is not justified.

Only:

- basic physicochemical testing;
- XRD;
- XRF;
- MTT

were performed.

Missing characterization includes potentially:

- particle size;
- SEM/TEM;
- elemental mapping;
- FTIR/Raman;
- ICP-MS/ICP-OES;
- surface chemistry;
- zeta potential where relevant;
- impurity profiling;
- toxicological characterization.

Required

Replace "fully characterised" with:

"characterized using selected pharmaceutico-analytical and in-vitro biological methods."

40. Lines 371–379 – Limitations

Positive aspect

The authors appropriately acknowledge several limitations.

However

The limitations should be expanded to include:

- absence/unclear positive control;
- absence of normal-cell cytotoxicity testing;
- uncertainty regarding MTT interference;
- unclear biological versus technical replicates;

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- lack of raw-data presentation;
- absence of quantitative impurity analysis;
- absence of dissolution/bioavailability data;
- absence of mechanism-of-action studies.

41. Lines 380–385 – Future perspectives

Positive point

The suggested future studies are reasonable.

However, the manuscript should not present mechanistic and animal studies merely as optional future work when the current evidence is being used to suggest therapeutic potential.

42. Conflict of interest

Lines 387–389

This section is acceptable, but the authors should also provide:

- funding information;
- institutional approval information;
- laboratory safety/ethical compliance where applicable.

43. Acknowledgements – Lines 392–399

Concern

The acknowledgement says:

"microbiological and heavy metal analyses"

but the Methods section does not adequately describe these analyses.

If heavy metal analysis was performed, the results should be included.

If it was not performed as part of the current study, this wording should be corrected.

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44. References – Lines 401–440

Major concern

The references require substantial correction.

Problems include:

- duplicate references;
- inconsistent citation details;
- incomplete bibliographic information;
- "Latest edition" without year;
- reference 16 appears to be **text copied from the manuscript rather than a genuine external reference**;
- inconsistent use of editions;
- unclear relationship between references and specific claims.

Particularly serious

Reference 16:

"The last Shadgunabalijarita Rasa Sindoor demonstrated organoleptic features…"

is not a conventional bibliographic reference. It appears to reproduce manuscript text.

This reference should be removed and replaced with the actual authoritative source supporting the relevant pharmacopoeial/organoleptic claim.

Additional major issue: ethical/safety documentation

Because the study involves human cancer cell lines, the authors should identify:

- source of A-549 cells;
- authentication;
- culture conditions;
- biosafety procedures.

Because the formulation contains mercury and potentially other toxic metals, the manuscript should also provide information regarding:

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- laboratory safety;
- handling of mercury-containing materials;
- disposal procedures;
- exposure control.

Most important reasons for MAJOR REVISION

If I were writing the formal editorial recommendation, I would summarize the major-revision decision around these **10 critical points**:

1. **The title and conclusions overstate the evidence** by describing the finding as "anti-lung cancer activity" rather than preliminary in-vitro cytotoxicity.
2. **The pharmaceutical methodology is insufficiently detailed** for independent reproducibility.
3. **Important batch-wise inconsistencies exist**, particularly in heating duration, maximum temperature and reported pharmaceutical data.
4. **The tables contain numerical inconsistencies**, especially the reported growth-inhibition percentages in Table 5.
5. **The MTT methodology is incomplete**, with inadequate information regarding cell density, exposure time, replicates, assay conditions and controls.
6. **Appropriate assay controls are not adequately described**, particularly positive control, vehicle control and formulation/MTT interference controls.
7. **The statistical analysis is insufficiently reported**, including lack of confidence intervals, replicate definition, p-values and dose-response model details.
8. **XRD/XRF characterization lacks essential methodological information and raw analytical presentation**, making independent verification difficult.
9. **The presence of potentially toxic elements such as Hg, Pb and As creates a significant safety concern**, and XRF alone is inadequate for definitive quantitative toxic-element assessment.
10. **Several claims exceed the data**, including "scientific proof," "fully characterized," "significant cytotoxic action," "safe/acceptable" implications, and therapeutic/anticancer potential.

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Minor but important editorial issues

The manuscript also needs extensive language and formatting revision:

- "pharmacological standardisation" should generally be "pharmaceutico-analytical standardization" where that is what was actually performed.
- "concentration dependant" → "concentration-dependent."
- "A-549" should be standardized throughout.
- "IC-50" → "IC50."
- "Rasa Sindoor"/"Rasa Sindura" terminology should be standardized.
- "pharmaceutico-analytical" should be used consistently.
- Table numbering should be corrected.
- Figure numbering should be checked.
- Scientific names and chemical formulas should be formatted consistently.
- "HgS in cinnabar phase" should be reported carefully as **α-HgS (cinnabar)** where supported by XRD.
- "mercuric sulphide" and "mercury sulphide" terminology should be standardized.
- "last Shadgunabalijarita…" in line 216 is clearly an error and should be "final product" or equivalent.
- "urge additional investigations" → "warrants further investigation."
- "The finding verifies…" should be replaced with more cautious language.

Suggested final reviewer recommendation

Recommendation: Major Revision

The manuscript presents an interesting integration of classical Rasashastra pharmaceutical processing, modern physicochemical characterization and preliminary in-vitro cytotoxicity evaluation. The topic has potential scientific and interdisciplinary significance. However, substantial revision is required before the manuscript can be considered for publication. The principal concerns include insufficient methodological detail for pharmaceutical preparation and cell-based experiments, inadequate description of analytical procedures, unclear definition of experimental versus technical replicates, insufficient statistical reporting, lack of adequately described assay controls, numerical inconsistencies in the cytotoxicity table, limited validation of XRF findings, and potentially important safety concerns related to mercury and other detected elemental impurities. In addition, several conclusions appear stronger than supported by the presented data. The authors should correct and verify all numerical data, provide raw/replicate-level experimental information where appropriate, strengthen analytical and statistical reporting, moderate claims regarding anticancer efficacy and safety, and substantially improve the reference list and manuscript presentation. Following satisfactory resolution of these issues, the

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study could provide useful preliminary evidence for further investigation of this traditional formulation.