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SHORT COMMUNICATION

The Drug-Drug Interactions (DDI) of 'Sharpunkhadi Granules with Hydroxyurea and Folic Acid' in HepG2 Cell Line - In Vitro Study

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ABSTRACT
This study evaluates the in vitro drug-drug interaction (DDI) of Sharpunkhadi granules (Ayurveda herbal formulation) in combination with hydroxyurea and folic acid using the HepG2 cell line. Sharpunkhadi granules were tested to assess their cytotoxicity and interaction with conventional drugs for managing conditions like sickle cell disease. The study employed different concentrations (100 mg/mL, 50 mg/mL, and 25 mg/mL) of Sharpunkhadi granules both alone and in combination with hydroxyurea and folic acid. The HepG2 cells were exposed to these combinations for 24 hours under controlled conditions (37°C, 5% CO2). Cytotoxicity and cell viability were assessed using microscopic examination and an MTT assay (Calorimetric assay to evaluate the cell toxicity). Results showed that all combinations had no cytotoxic effect, with cell viability consistently above 70%, indicating no adverse interactions or synergistic toxicity. These findings suggest that Sharpunkhadi granules can be safely co-administered with hydroxyurea and folic acid without altering their pharmacological safety and efficacy.

Keywords; *Drug-drug Interaction, DDI, Sharpunkhadi Granules, In-vitro, Hydroxyurea, Folic Acid*

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INTRODUCTION

Drug-drug interactions (DDIs) are a significant concern in modern and traditional medicine, as they can impact therapeutic outcomes by altering the pharmacodynamics or pharmacokinetics of the drugs involved. In recent years, there has been a growing interest in understanding the interactions between conventional pharmaceuticals and Ayurvedic formulations, especially in the context of chronic diseases like sickle cell disease (SCD). (1,2) SCD is a hereditary blood disorder that often requires management with hydroxyurea, a drug known to increase fetal hemoglobin production and reduce the frequency of vaso-occlusive crises. (3,4) Additionally, folic acid is commonly prescribed to SCD patients to counteract anemia and support red blood cell production. (5) Sharpunkhadi granules, an Ayurvedic formulation known for its blood-purifying and anti-inflammatory, anti-oxidant, anti-sickling, and rejuvenating properties are traditionally used in managing blood disorders specifically anemias. However, little is known about the safety and interaction profile of Sharpunkhadi granules when used in combination with modern drugs like hydroxyurea and folic acid. To address this knowledge gap, this study aimed to evaluate the in vitro interactions of Sharpunkhadi granules with these two drugs using the HepG2 liver cell line, which is commonly used to study drug metabolism and cytotoxicity. (6) This study provides valuable insights into the compatibility of combining traditional Ayurvedic formulations with conventional pharmaceuticals by assessing the potential cytotoxic effects and cell viability through

microscopic examination and MTT assay. The findings are particularly relevant for patients undergoing polypharmacy to treat chronic conditions like sickle cell disease.

MATERIAL AND METHODS

STUDY DESIGN:

This study evaluates in vitro Drug-Drug Interactions (DDIs) of "Sharpunkhadi Granules" with Hydroxyurea and Folic Acid using the HepG2 cell line. (7) The study was conducted following Good Laboratory Practices (GLP) as per applicable guidelines. (8)

STUDY DESIGN/PROTOCOL NUMBER: RRC/NG/24/PT/0037

TEST ITEMS:

The Sharpunkhadi Granules, Hydroxyurea, and Folic Acid used in this study were supplied by Parul Institute of Ayurved, Parul University. Detailed compositions, batch numbers, and other characteristics were provided. Solubility tests indicated that Sharpunkhadi Granules were soluble at a concentration of 100 mg/mL.

Sharpunkhadi Granules (Batch No. PIAP23001): Manufactured on 03/06/2023, with an expiry date of 02/06/2025. The physical state was solid and stored at room temperature.

Hydroxyurea Capsules IP (500 mg): Batch CWZ1183, manufactured 12/2023, expiry 11/2025.

Folic Acid Tablet IP (5 mg): Batch GCH1111, manufactured 10/2023, expiry 09/2025.

TEST SYSTEM

Cell Line: HepG2, a liver tissue-derived human cell line, was used. The complete growth medium used was Minimum Essential Medium (MEM) supplemented with 10% Fetal Bovine Serum (FBS), sodium bicarbonate, 100 IU/mL penicillin, and 100 µg/mL streptomycin. The culture conditions were 5% CO_2, 37°C, and ≥90% humidity.

Rationale for Test System: The HepG2 cell line was selected for drug interaction studies due to its established use in liver metabolism studies.

Preparation of Test System:

HepG2 cells were cultured in MEM medium until they reached the appropriate confluence.

Cells were detached with 0.25% trypsin-EDTA and counted using a hemocytometer.

The concentration was adjusted to 1.09×10^5 cells/mL, and 100 µL of this suspension was seeded into each well of a 96-well plate.

The cells were incubated for 24 hours to form a half-confluent monolayer before exposure to the test items.

Experimental Groups:

Three concentrations of Sharpunkhadi Granules were tested:

a. 100 mg/mL
b. 50 mg/mL
c. 25 mg/mL

Each concentration was tested alone and in combination with: Hydroxyurea 500 mg and Folic Acid 5 mg, and together with both.

Control groups included a vehicle control (MEM media), and positive controls with Hydroxyurea or Folic Acid alone.

Exposure Protocol:

After removing the culture medium from the wells, 100 µL of the prepared test item, vehicle, or controls were added to each well in triplicate. The cells were incubated for another 24 hours at 37°C, 5% CO_2, and ≥90% humidity.

Microscopic Examination:

The treated cells were examined under an inverted microscope after 24 hours to observe morphological changes, such as cell detachment, vacuolization, and cell lysis. The qualitative grading of cytotoxicity was assigned based on the morphological characteristics using a grading scale from 0 (no reactivity) to 4 (severe cytotoxicity).

MTT Assay (9,10, 11)

To evaluate cell viability, the MTT assay was performed:

a) 10 mg MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) was dissolved in 10 mL MEM and sterilized using a 0.22 µm filter.
b) After exposure, the MTT reagent (50 µL) was added to each well, and cells were incubated for 2 hours.
c) 100 µL isopropanol was added to each well to solubilize the formazan crystals.

d) The optical density (OD) was measured at 570 nm using a microplate reader. The cell viability percentage was calculated using the following formula:

$$\text{Cell Viability (\%)} = \text{OD of Treated} / \text{OD of Control} \times 100 \quad (12,13)$$

Data Analysis:
The results were analyzed using both qualitative morphological grading and quantitative biochemical assays based on cell viability. A cell viability of >70% indicated non-cytotoxicity.

Statistical Analysis:
All data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using appropriate software tools. Significance was determined at $P<0.05$ for comparative analysis.

Safety and Quality Control:
Personnel adhered to safety protocols, including wearing PPE (gloves, face masks, head caps). All procedures followed Standard Operating Procedures (SOPs) established by the test facility.

RESULTS

The microscopic examination provided qualitative insights into the morphological integrity of HepG2 cells following exposure to various concentrations of Sharpunkhadi Granules, both alone and in combination with Hydroxyurea and Folic Acid. The HepG2 cells were observed for several cytotoxic indicators, including: There was no significant cell detachment was noted across all treatment groups. Whereas, Vacuolization and Cell Swelling, the signs of cellular distress were absent in cells exposed to the test item and control groups. Cell Lysis and Membrane Integrity cell membrane remained intact for all treatments. No evidence of cell lysis or membrane rupture was observed. All three concentrations of Sharpunkhadi Granules (100 mg/mL, 50 mg/mL, and 25 mg/mL) demonstrated no adverse morphological changes when compared to the vehicle control. Furthermore, when combined with Hydroxyurea and Folic Acid, cells maintained their normal morphology, indicative of non-cytotoxic behavior. These results align with a reactivity grade of 0 (None) for all test groups, which confirms the absence of cytotoxic reactivity at all test concentrations. The combination of Sharpunkhadi Granules with Hydroxyurea and Folic Acid similarly showed no detrimental effect on cellular morphology. Cells exposed to these combinations were visually indistinguishable from those in the vehicle control group, further confirming the non-cytotoxic nature of the test items and combinations.

MTT Assay: Cell Viability Assessment:
The MTT assay quantitatively assessed cell viability based on the conversion of MTT to formazan crystals by metabolically active cells. The formation of purple formazan crystals indicates the presence of viable cells, and the intensity of the purple color correlates with the number of live cells in each well. (14) Table 1 summarizes the optical density (OD) values at 570 nm and the corresponding cell viability percentages for each experimental group.

Table 1: MTT Assay - Cell Viability Results (OD at 570 nm and % Cell Viability)

Experimental Group	Replicate 1 OD	Replicate 2 OD	Replicate 3 OD	Average OD	% Cell Viability
Vehicle Control	0.360	0.381	0.324	0.355	100%
Positive Control (Hydroxyurea)	0.389	0.414	0.379	0.394	111%
Positive Control (Folic Acid)	0.322	0.328	0.331	0.327	92%
Sharpunkhadi Granules (100 mg/mL)	0.350	0.372	0.312	0.345	97%
Sharpunkhadi Granules (50 mg/mL)	0.362	0.338	0.325	0.342	96%
Sharpunkhadi Granules (25 mg/mL)	0.315	0.349	0.348	0.337	95%
Sharpunkhadi Granules + Hydroxyurea (100 mg/mL)	0.326	0.348	0.365	0.346	98%
Sharpunkhadi Granules + Folic Acid (100 mg/mL)	0.354	0.370	0.387	0.370	104%
Sharpunkhadi Granules + Hydroxyurea + Folic Acid	0.366	0.364	0.354	0.361	102%

Individual Treatment Groups:
In Sharpunkhadi Granules Alone the test concentrations of Sharpunkhadi Granules (100 mg/mL, 50 mg/mL, and 25 mg/mL) did not demonstrate any significant reduction in cell viability. The % cell viability remained consistently above 95% across all concentrations, with the highest concentration (100 mg/mL) showing a viability of 97%. This result suggests that Sharpunkhadi Granules do not exhibit cytotoxic effects on HepG2 cells under the tested conditions. (15)
While the Sharpunkhadi Granules and Hydroxyurea combined, the cell viability percentages were slightly higher, with the 100 mg/mL combination yielding 98% cell viability. This indicates that the combination does not introduce additional cytotoxicity compared to either Sharpunkhadi Granules or Hydroxyurea alone. The combinations of Sharpunkhadi Granules and Folic Acid showed similar results, with viability percentages consistently above 100%. The highest cell viability was observed in the 100 mg/mL Sharpunkhadi Granules + Folic Acid combination, which recorded 104% cell viability. This slightly elevated viability suggests the non-toxic interaction between these agents. The triple combination of Sharpunkhadi Granules, Hydroxyurea, and Folic Acid resulted in a cell viability percentage of 102%, indicating that the combined use of all three substances does not lead to a synergistic or toxic effect. The cell viability remained above the cytotoxic threshold of 70% across all replicates, signifying the safety of co-administration.

Cytotoxicity Threshold and Safety Evaluation:
A cytotoxic effect is typically defined when cell viability drops below 70%, which serves as a standard threshold in vitro cytotoxicity assessment. In this study, none of the tested concentrations—whether Sharpunkhadi Granules were used alone or in combination—exhibited viability below this threshold. The absence of cytotoxicity across all experimental groups is reflected both qualitatively (through morphological assessment) and quantitatively (via MTT assay).

Comparison with Positive Controls:
As expected, the positive control group treated with Hydroxyurea (500 mg/mL) demonstrated an elevated cell viability of 111%, suggesting a potential effect on the HepG2 cells in this in vitro model. However, this effect was not deemed synergistic when combined with Sharpunkhadi Granules or Folic Acid, as the combinations displayed similar viability levels without enhancing cytotoxicity.
Treatment with only Folic Acid (5 mg/mL) resulted in a cell viability of 92%, indicating that Folic Acid alone does not significantly impact HepG2 cell survival at this concentration. When combined with Sharpunkhadi Granules, the results remained consistent, reinforcing the conclusion that the combination is safe for use.

Overall Summary of Results:
- Non-cytotoxicity: Sharpunkhadi Granules, both individually and in combination with Hydroxyurea and Folic Acid, exhibited no cytotoxic effects on HepG2 cells.
- Cell Viability: Cell viability percentages remained above 70% across all tested conditions, suggesting that these agents do not adversely affect cell viability.
- No Synergistic Toxicity: The combination of Sharpunkhadi Granules with Hydroxyurea and Folic Acid did not result in any synergistic toxicity or interaction, as cell viability percentages remained high.
- Morphological Integrity: Microscopic examination confirmed that there were no significant changes in cell morphology, further supporting the non-cytotoxic findings from the MTT assay.

Table No 2: Cell Viability Data

Experimental Group	% Cell Viability
Vehicle Control	100%
Positive Control (Hydroxyurea)	111%
Positive Control (Folic Acid)	92%
Sharpunkhadi Granules (100 mg/mL)	97%
Sharpunkhadi Granules + Hydroxyurea (100mg)	98%
Sharpunkhadi Granules + Folic Acid (100mg)	104%

DISCUSSION
The in vitro study on *Sharpunkhadi* Granules in combination with Hydroxyurea and Folic Acid revealed crucial insights into potential drug-drug interactions, using the HepG2 liver cell line as the test system. The results are significant in understanding the cytotoxic effects and possible pharmacological interactions when these substances are co-administered. The primary goal of this study was to assess

whether the combination of these three substances (*Sharpunkhadi* Granules, Hydroxyurea, and Folic Acid) would exhibit any synergistic or antagonistic effects on cell viability and cytotoxicity.

The study produced several important findings:

Non-cytotoxicity of *Sharpunkhadi* Granules: *Sharpunkhadi* granules, at all tested concentrations (100 mg/mL, 50 mg/mL, and 25 mg/mL), showed no cytotoxicity to the HepG2 cells. This was demonstrated through microscopic examinations, where no abnormal changes in cell morphology, such as detachment, vacuolization, or membrane disruption, were observed. These findings are crucial, as they suggest that *Sharpunkhadi* Granules do not adversely affect liver cells at the tested concentrations. (16, 17)

Non-reactivity in Combinations: The study also tested the combination of *Sharpunkhadi* Granules with Hydroxyurea and Folic Acid. Both combinations showed no reactive changes under the microscope, confirming that these substances do not interfere with each other in ways that affect HepG2 cells. This finding is significant for clinical applications, where the concurrent use of traditional herbal remedies like *Sharpunkhadi* Granules and conventional pharmaceuticals is common, especially in managing chronic conditions such as Sickle Cell Disease.

MTT Cell Viability Assay Results: Quantitative biochemical assays using the MTT method further supported the qualitative findings. All combinations of the test substances showed cell viability greater than 70%, indicating that none of the substances, either alone or in combination, caused significant cytotoxicity to HepG2 cells. This suggests that the concurrent use of these substances does not adversely affect liver cell function.

Implications for Drug-Drug Interactions (DDIs): The absence of cytotoxic effects or adverse morphological changes implies that the use of *Sharpunkhadi* granules with Hydroxyurea and Folic Acid does not lead to harmful drug-drug interactions. This is especially important considering Hydroxyurea's common use in managing Sickle Cell Disease and Folic Acid's role in preventing anaemia. The study's findings suggest that *Sharpunkhadi* granules can be safely combined with these drugs without exacerbating liver toxicity. (18, 19)

CONCLUSION

The study successfully demonstrated that Sharpunkhadi granules, alone and in combination with Hydroxyurea and Folic Acid, exhibit no cytotoxic effects on HepG2 liver cells. Both qualitative and quantitative analyses indicate that Sharpunkhadi Granules is non-reactive and non-cytotoxic, with cell viability greater than 70% across all tested concentrations. The absence of observable drug-drug interactions suggests that Sharpunkhadi Granules may be safely used alongside Hydroxyurea and Folic Acid, making it a promising adjunctive treatment for conditions like Sickle Cell Disease. (20, 21) Further studies, particularly in vivo, are recommended to confirm these findings in more complex biological systems. (22, 23, 24)**.** The results from this study contribute valuable data to the understanding of traditional herbal medicines in modern therapeutic regimens, offering evidence for the safe integration of Sharpunkhadi Granules into clinical settings.

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