

Domestic preparation of *Hygrophila spinosa* (Kulekhara) decoction mitigates iron deficiency anemia and restores erythropoiesis in an experimental murine model

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ABSTRACT

Background: The failure of the anemia eradication program and its inefficacy underscore the need for alternative therapeutic strategies, including herbal remedies. **Objectives:** This study evaluates the hematinic effects and erythropoiesis in iron deficiency anemia (IDA) of domestically prepared and consumed *Hygrophila spinosa* (Kulekhara) decoction (KKD). **Methods:** KKD was prepared by boiling the human equivalent dose in water till the volume was halved. An experimental IDA model was developed with the low-iron diet for a month. Once anemia was established, animals were divided into three groups, namely IDA receiving the vehicle only, IDA+Iron Supplement group with iron supplement, and IDA+KKD group receiving KKD for 2 weeks. After sacrificing, hematological parameters and iron indices were analyzed. The hematinic potential of KKD was assessed through immunophenotypic classification of bone marrow hematopoietic progenitors using Ter119 and CD71 markers. **Results:** Total hemoglobin concentration, plasma iron levels, and Ter119 and CD71 expression in bone marrow were reduced in IDA and restored significantly in iron supplementation and KKD treatment, with KKD showing better recovery than an iron supplement. **Conclusion:** The findings of this study indicate that Kulekhara decoction represents a promising alternative for managing iron deficiency anemia.

Keywords: Iron deficiency, Anemia, *Hygrophila spinosa*, Kulekhara, Erythropoiesis, Herbal medicine, Ethnomedicine.

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INTRODUCTION

Anemia is a major global public health issue.¹ About one-third of all women of reproductive age suffer from anemia, with iron deficiency accounting for approximately 51% of these cases.² Iron deficiency anemia (IDA) is the most prevalent form of anemia, accounting for approximately 50% of all anemia cases and primarily affecting children, adolescents, and pregnant women.³ India carries a significantly large burden of anemia.⁴

Iron restriction and impaired iron supply for erythropoiesis in bone marrow lead to erythroid hypoproliferation and, consequently, IDA.⁵ As iron is crucial for effective erythropoiesis to generate erythrocytes, absolute or true iron deficiency makes the metal unavailable for the proper differentiation and maturation of erythropoietic progenitor cells, ultimately leading to a reduction in erythron production, causing anemia.⁶

Iron deficiency anemia may coexist with a chronic inflammatory condition.⁷ Hence, treatment of anemia requires diagnosis of the underlying cause. Despite several governmental anemia eradication programs distributing free oral supplemental iron, the prevalence rate of IDA in India has increased.⁸ The ineffectiveness of iron supplementation might partially have resulted from poor acceptability, probably due to the associated side effects such as nausea, vomiting, constipation, stomach pain, etc. Moreover, lower gastrointestinal absorption of iron, increased free radical production, and oxidative stress have contributed to the failure to improve the prevalence of anemia.⁹

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In the Indian context, where herbal remedies are ingrained in the culture and popular due to their cost-effectiveness, *Hygrophila spinosa* (Kulekhara), an anti-anemia herb, holds high potential for acceptability and can therefore be easily integrated into the lifestyle of anemia patients for long-term relief from anemia symptoms. Kulekhara is a popular herbal medicine for its antipyretic, hepatoprotective, diuretic, anti-inflammatory, and hematopoietic effects.¹⁰⁻¹³ Despite several scientific studies on the ethanolic extract of Kulekhara

leaves¹⁴⁻¹⁶ no report is available on the effectiveness of the aqueous decoction of leaves, which is the most popular form in which people consume it in anemia. The present study focuses on understanding the hematinic effect and erythropoietic effect of Kulekhara decoction in an experimental murine model.

MATERIALS AND METHODS

Animals

Adult female Swiss albino mice (n=24), aging around 12 weeks and weighing about 26 to 32 g, were bought from an authorized mouse breeder. They were housed under a regulated ambience (Temperature $22 \pm 4^\circ\text{C}$, relative humidity $60 \pm 5\%$) and received a balanced diet and water *ad libitum*. The study protocol was approved by the Institutional Animal Ethical Committee, Department of Physiology, University of Calcutta, which endorsed the current animal experiments following the guidelines of the Committee for the Control and Supervision of Experiments on Animals, Government of India (Ethical no- IAEC-V/T/RM-04 (Anusua Singh)/2019 date 7.8.19).

Preparation of Kulekhara decoction (KKD)

Fresh *H. spinosa* (Kulekhara) leaves were collected from a local market in Kolkata, West Bengal, India. The plants were morphologically identified from the Botanical Survey of India, Central National Herbarium, Howrah, Kolkata, West Bengal, India, with reference number CNH/Tech.II/2018/94. The leaves of Kulekhara were cleaned thoroughly with distilled water and dab-dried and used for further study. The amount of Kulekhara leaf usually taken by humans was converted to a human equivalent dose in mice,¹⁷ and accordingly, a 2 g/kg body weight dose was selected. The decoction was prepared from fresh Kulekhara leaves boiled in water till the volume was halved. The preparation was then strained and stored in a glass vial for further studies. The decoction was prepared freshly daily for the animal study.

Development of an iron deficiency anemia model

The mice were equally divided into four groups: Group 1, Control; Group 2, IDA; Group 3, IDA + Iron Supplement; and Group 4, IDA + KKD, each group consisting of six mice. The iron deficiency anemia model was developed by administering a low-iron diet and deionized water for one month. The onset of anemia was defined as a reduction in total hemoglobin content by ≥ 2 g/dL, a decrease in hematocrit percentage, and an increase in total iron-binding capacity.²⁷ After confirmation of anemia development, the treatment groups were administered an iron supplement equivalent to the human dose^{17,18} and KKD orally for 2 weeks, respectively. In contrast, the disease control group was fed only the vehicle, deionized water (Figure 1).

Blood and tissue collection

Animals were sacrificed after the end of the treatment period, and blood was drawn from the heart and collected

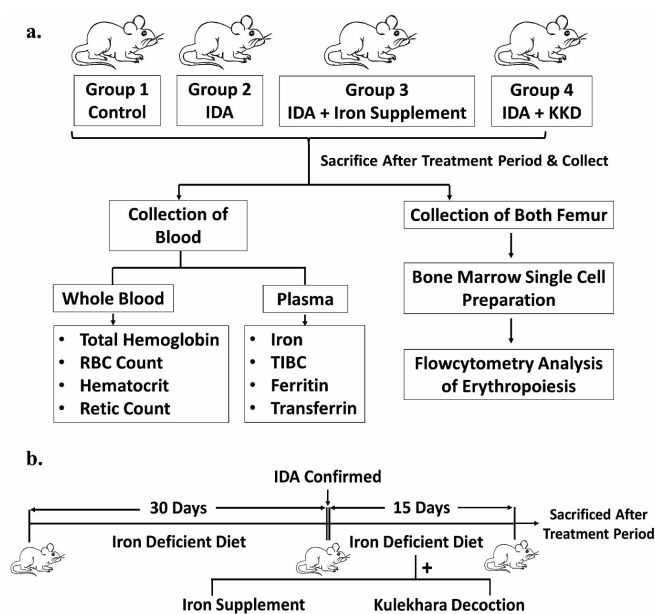


Figure 1: The (a) study design and (b) timeline of iron deficiency anemia model development in mice and treatment with iron supplementation and Kulekhara decoction

in a heparinized vial. Whole blood was used to measure the hematological parameters, and plasma was collected after centrifugation of whole blood (5000 rpm, 5 min) and used for biochemical assays. Both femurs were harvested aseptically for flow cytometry analysis of erythropoiesis.

Estimation of hematological parameters

The total hemoglobin (Hb) content was evaluated spectrophotometrically by using Drabkin's Kit method.¹⁹ The red blood cell (RBC) count was performed using an improved Neubauer counting chamber.²⁰ The Hct and reticulocyte count were done manually, according to Lewis *et al.*²¹

Estimation of iron and total iron-binding capacity (TIBC) in plasma

Plasma iron and TIBC were measured using the ferrozine method using a commercially available biochemical kit (Iron & TIBC Kit, Ferrozine/Magnesium Carbonate Method, Lot No: RIRT1103, Coral Clinical System, Goa, India) according to the manufacturer's guidelines.

Estimation of plasma ferritin and transferrin

Ferritin (Catalogue No: EM1019, Fine Test, Wuhan, China) and transferrin were measured using an ELISA kit according to the manufacturer's guidelines (Catalogue No: KSC036Mu11, Cloude Clone Corp, USA).

Bone marrow single-cell preparation

The femur was aseptically excised and placed in a petri dish containing 0.01 M PBS, pH 7.4. For the isolation of bone marrow (BM) cells, the femurs were flushed with PBS containing 0.2% bovine serum albumin (BSA) and centrifuged at 10000 rpm at room temperature for 5 minutes. Afterward,

the supernatant was discarded, and the resulting pellet was washed with PBS until a clear white pellet was obtained. It was then resuspended in 0.01 M PBS, pH 7.4, for further processing. The total number of BM cells was counted using a Neubauer chamber after appropriate dilution and recorded.

Cell surface marker staining and flow cytometry analysis of bone marrow cells

The viability assay of resuspended BM cells was assessed using trypan blue dye, yielding more than 95% viable cells in both cases. The collected BM cells were resuspended in PBS solution at a concentration of $\sim 10^6$ cells/100 μ L for staining with Ter119-PE-Cy7 (Material No: 557853, PE-CyTM 7 Rat Anti-Mouse TER-119/Erythroid Cells BD PharmingenTM, San Jose, CA, USA) and CD71-PE (Material No: 567206, PE Rat Anti-Mouse CD71 BD PharmingenTM, San Jose, CA, USA) for 30 min at 2 to 8°C and washed with 0.5% PBS-BSA for removal of unbound antibody/reagent and were resuspended in 0.01 M PBS, pH 7.4. Stained cells were analyzed with BD FACS Lyric flow cytometer, and the data were analyzed using FlowJo version 10.10.²²

Statistical Analysis

The results were represented as the mean $\pm$ standard deviation of the mean and as percentages or frequencies as required. The normality of the data distribution was checked, and one-way ANOVA followed by Tukey HSD *post hoc* analysis was performed to check any statistical difference between the studied groups. A two-tailed $p < 0.05$ was considered statistically significant. All calculations were performed using Microsoft Excel 2019 and OriginPro 2024.

RESULTS

Changes in hematological parameters of the IDA model treated with KKD

The IDA was confirmed by a decrease in total Hb concentration of more than 2 g/dL, Hct, and plasma iron content, accompanied by an increase in TIBC, compared to the control group. After the establishment of the IDA model,

the animals were treated with iron and KKD. The baseline Hb levels of the four studied groups were measured and compared before and after the treatment intervention. No significant change was observed in the control group. In the IDA group, a significant ($p < 0.05$) reduction of 5 g/dL in the Hb concentration was observed after treatment with a low-iron diet. The iron supplementation showed recovery after the intervention; however, the Hb content was found to be significantly ($p < 0.05$) lower than the baseline value. Contrarily, KKD showed an insignificant difference with the baseline data after the intervention, showing that it was able to correct the IDA. The hematological parameters, including total Hb content, total RBC count, Hct percentage, and reticulocyte count, were compared among the four groups and demonstrated statistically significant differences between the measured variables (Table 1). The iron supplementation partially restored Hb levels, whereas KKD treatment showed slightly better anemia correction than the iron supplement, with total Hb approaching control values. A similar feature was observed in RBC counts, where IDA significantly ($p < 0.05$) decreased RBC numbers compared to the control group. KKD treatment was more effective than conventional iron in improving RBC counts. A comparable pattern was observed in the hematocrit (Hct) level, with IDA inducing a significant decline ($p < 0.05$) compared to the control. KKD administration markedly improved Hct, showing better recovery than iron supplementation. The reticulocyte percentage was significantly ($p < 0.05$) reduced in the IDA group compared to the control. Both treatment modalities increased reticulocyte counts, with KKD showing a better stimulatory effect compared to iron supplements.

Changes in iron parameters of the IDA model treated with KKD

To investigate the alterations in iron metabolism induced by iron deficiency and evaluate the therapeutic intervention of KKD, plasma iron, total iron-binding capacity (TIBC), ferritin, and transferrin were assessed across all experimental groups (Table 1). Plasma iron concentration was found to be significantly ($p < 0.05$) reduced in the IDA group compared to

Table 1: Changes observed in the hemogram and iron profile of IDA treated with Kulekhara decoction

	Control	IDA	IDA+Iron Supp	IDA+KKD
Hemoglobin (g/dL)	15.41 $\pm$ 0.37*	10.00 $\pm$ 0.84* ^{@#}	13.21 $\pm$ 0.19 [@]	14.93 $\pm$ 0.56 [#]
RBC Count ($\times 10^6/\mu$ L)	10.23 $\pm$ 0.95*	8.23 $\pm$ 0.41* ^{@#}	8.99 $\pm$ 0.65 [@]	10.49 $\pm$ 1.66 [#]
Hematocrit (%)	50.13 $\pm$ 0.94*	32.21 $\pm$ 1.58* ^{@#}	43.34 $\pm$ 2.03 [@]	48.01 $\pm$ 2.7 [#]
Reticulocyte Count (%)	1.93 $\pm$ 0.25*	0.47 $\pm$ 0.19* ^{@#}	1.10 $\pm$ 0.20 [@]	1.48 $\pm$ 0.19 [#]
Iron (μ g/dL)	223.59 $\pm$ 5.37*	73.63 $\pm$ 3.52* ^{@#}	159.54 $\pm$ 6.21 [@]	176.39 $\pm$ 2.57 [#]
TIBC (μ g/dL)	417.27 $\pm$ 7.26*	598.77 $\pm$ 2.64* ^{@#}	297.46 $\pm$ 5.24 [@]	327.94 $\pm$ 4.27 [#]
Ferritin (ng/mL)	149.38 $\pm$ 2.45*	48.71 $\pm$ 4.23* ^{@#}	102.30 $\pm$ 4.85 [@]	124.71 $\pm$ 3.12 [#]
Transferrin (mg/L)	7.99 $\pm$ 0.32*	13.38 $\pm$ 1.16* ^{@#}	8.70 $\pm$ 0.55 [@]	7.78 $\pm$ 1.00 [#]

Data are expressed as mean $\pm$ SD, n = 6, significance level $p < 0.05$ in * control vs IDA, @ IDA vs IDA+Iron Supp, and # IDA+KKD. IDA- Iron deficiency anemia, IDA+Iron Supp- IDA treated with iron supplement, and IDA+KKD- IDA treated with Kulekhara decoction.

controls. Both treatments improved the iron concentration, with $159.54 \pm 3.58 \mu\text{g/dL}$ achieved with iron supplement and $176.39 \pm 1.48 \mu\text{g/dL}$ with KKD, indicating better restoration of systemic iron availability with KKD. TIBC was significantly ($p < 0.05$) elevated in the IDA group compared to the control. Both treatment groups reduced TIBC, with KKD showing slightly better correction than conventional iron therapy. Ferritin was drastically reduced in the IDA group compared to controls. IDA correction with KKD treatment was better compared to iron supplementation. The serum transferrin level was significantly ($p < 0.05$) elevated in the IDA group compared to the control. Both treatment modalities reduced transferrin concentrations, with KKD and iron supplementation.

Erythrocyte maturation in bone marrow

To assess the impact of KKD on erythropoiesis in the IDA model, we analyzed the expression of erythroid lineage marker Ter119 and cell proliferation marker CD71 on bone marrow cells. We observed the changes in erythroid maturation stages. In the iron-deficient group, the frequency of Ter119+ cells was significantly ($p < 0.05$) reduced (from $41.8 \pm 6.29\%$ in control to $17.46 \pm 0.58\%$ in IDA, $p < 0.05$), indicating a marked erythroid suppression under iron-deficient conditions. Iron treatment restored the Ter119+ population to near-normal levels ($44.83 \pm 5.14\%$, $p > 0.05$ vs. control). In contrast, KKD treatment led to more pronounced restoration of Ter119+ cells ($52.43 \pm 1.60\%$, $p < 0.01$ vs. IDA) compared to iron supplementation (Figure 2 ai-aiv). Notably, a 3-fold increase in the Ter119+ percentage was observed in the KKD group compared to IDA, suggesting a complete recovery of erythroid lineage output, and even slightly better than the iron-treated group, which showed near-complete but not full restoration (Figure 2c and d). The pro-erythroblast (ProE) population, the early stage of erythropoiesis, showed an increase in the IDA group compared to control ($1.30 \pm 0.48\%$ vs. $0.39 \pm 0.08\%$ in control, $p > 0.05$), which was reduced after iron and KKD treatment. However, the changes observed in the disease and treatment groups were marginally insignificant (Figures 2e and 2f). To understand the effect of KKD in erythroid maturation, the Ter119+ cells were classified into three stages based on CD71 expression- EryA, EryB, and EryC. There was a distinct alteration observed in the expression of these three stages across the experimental groups (Figure 2 bi-biv). The percentage population of EryA was significantly ($p < 0.01$) increased in the IDA ($26.53 \pm 1.36\%$) group compared to the control ($12.23 \pm 0.82\%$). However, the absolute count was reduced in IDA (3.94 ± 0.67 to $2.85 \pm 0.44 \times 10^6$, $p < 0.05$), suggesting compensatory accumulation of early erythroid progenitors, which contributed to the reduced expression of later stages (Figure 2e and f). It was observed that the EryA level was significantly ($p < 0.05$) corrected after treatment interventions. The iron and KKD treatment significantly increased EryA expression to $6.84 \pm 0.44 \times 10^6$ and $6.11 \pm 1.38 \times 10^6$ ($p < 0.05$ vs. IDA), respectively, whereas it reduced to $15.53 \pm 2.14\%$ in IDA ($p < 0.05$ vs. IDA). EryB population was significantly reduced in IDA (from 12.35

$\pm 4.01 \times 10^6$ in control to $2.63 \times 10^6 \pm 0.58 \times 10^6$ in IDA, $p < 0.05$), indicating impaired maturation. Both treatments significantly increased the level of EryB expression, to $14.71 \pm 1.84 \times 10^6$ with iron and to $17.62 \pm 1.35 \times 10^6$ with KKD (for both treatments, $p < 0.05$ vs. IDA), reflecting effective anemia correction. The absolute count of Ery-C, representing the terminal stage, also decreased significantly ($p < 0.05$) in IDA ($4.00 \pm 0.44 \times 10^6$) relative to control ($13.81 \pm 1.8 \times 10^6$). While iron treatment increased EryC to $9.69 \pm 1.37 \times 10^6$, KKD led to a significantly higher recovery ($13.05 \pm 0.77 \times 10^6$, $p < 0.05$ vs. IDA), indicating KKD was more effective in correcting erythropoiesis (Figure 2e and 2f).

DISCUSSION

Anemia, the most prevalent global health issue, significantly decreases the quality of life and reduces economic productivity.²³ Iron deficiency anemia is the most common form of anemia, affecting 1.62 billion people worldwide.^{24,25} The conventional treatment for iron deficiency anemia primarily involves oral iron supplementation, which is associated with several side effects, including nausea, constipation, abdominal pain, and diarrhea, leading to poor patient compliance and ultimately limiting the effectiveness of iron supplementation programs such as the Anemia Mukh Bharat program.²⁶ The high prevalence of IDA and the limitations of conventional treatments led to alternative therapies, particularly traditional medicine, including herbs. Herbal remedies have been used since ancient times to treat several ailments, including anemia. *Hygrophila spinosa*, also known as Kulekhara, is one such herbal remedy with well-supported ethnobotanical evidence of its role in correcting anemia.¹³ This study provides scientific evidence supporting the traditional use of Kulekhara in the treatment of iron deficiency anemia, shedding light on its mechanisms of action.

In the present study, we observed that KKD significantly corrects Hb levels, RBC count, reticulocyte count, and Hct in iron-deficient mice. These observations align with traditional claims regarding the hematinic properties of Kulekhara and provide a scientific basis for its use in treating anemia. Studies have shown that the ethanolic extract of the aerial parts of Kulekhara significantly reduces the anemic condition after treatment.¹⁴⁻¹⁶ A study conducted by Nagababu *et al.*[27] demonstrated that a reduction in hemoglobin level to nearly 10.5 g/dL, along with a decrease in hematocrit percentage from 50 to 40%, could be indicative of iron deficiency anemia. In our study, when the IDA group was treated with Kulekhara herbal decoction, the hemoglobin level increased by more than 30% compared to the anemic animals. Similarly, a greater than 20% increase was observed in the RBC count, suggesting that the hematological parameters were significantly altered after treatment. The Hct level and reticulocyte count also normalized after treatments, with KKD showing better recovery than an iron supplement. Studies indicate that in anemia of different etiologies, Kulekhara can

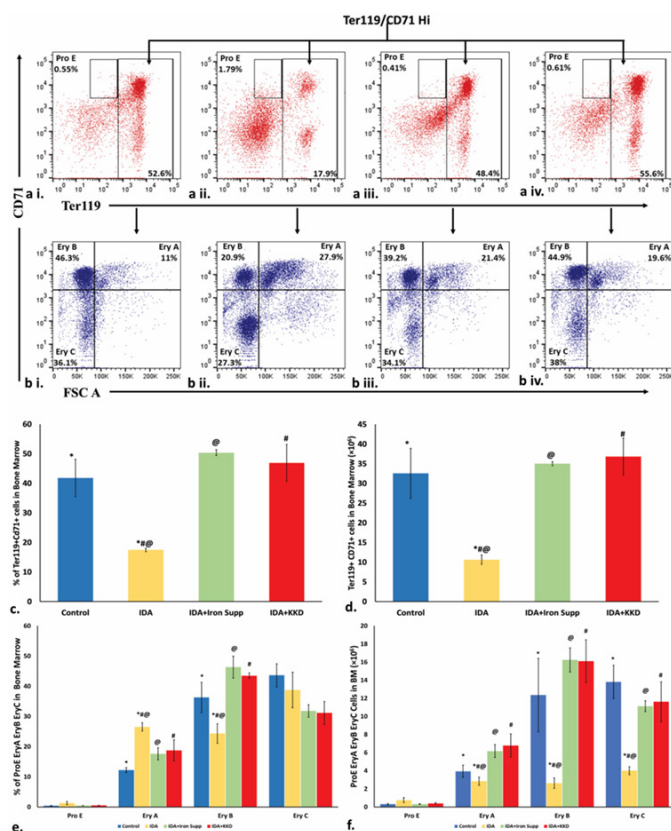


Figure 2: Effect of ethnomedicinal herbal decoction of *Hygrophila spinosa* (Kulekhara) on erythropoiesis in iron deficiency anemia model. (a) Expression of Ter119/CD71 positive cells and ProE and (b) classification of EryA, EryB, EryC in bone marrow of control (i), IDA (ii), IDA+Iron Supp (iii) and IDA+KKD (iv) treatment group. (c) Percentage and (d) number of Ter119/CD71 positive cells in bone marrow of the studied groups. (e) Percentage and (f) number of ProE, EryA, EryB and EryC in bone marrow of the studied groups. Data were expressed as Mean $\pm$ SE. * indicates statistical significance of $p < 0.05$ between control vs IDA, @ indicates statistical significance of $p < 0.05$ between IDA vs IDA+Iron Supp and # indicates statistical significance of $p < 0.05$ between IDA vs IDA+KKD.

reverse the anemic condition by altering the hemogram. The ethanolic extract of Kulekhara significantly reversed the anemic condition by improving hemoglobin content and other hematological parameters¹⁴. The findings were further supported by a study in which the ethanolic extract of Kulekhara effectively restored hematological parameters in haloperidol-induced iron deficiency anemia, suggesting its hematinic properties.¹⁵ In phenyl-hydrazine hydrochloride-induced iron deficiency anemia, the aqueous extract of *Hygrophila spinosa* restores the hemogram profile, confirming that it stimulates the hemopoietic system²⁸. Although they used the aqueous extract, their study did not mention the method of preparation of Kulekhara. In the present study, we used the Kulekhara decoction, prepared in the traditional manner, which demonstrated greater efficacy in reversing IDA. These findings align with our results, where hemoglobin and RBC count were positively restored in IDA after treatment with Kulekhara herbal decoction. Significant improvements in

plasma iron and TIBC were observed. Ferritin and transferrin are two major proteins that maintain iron homeostasis in the body. Ferritin is the iron storage protein, while transferrin is the main iron transporter protein that distributes iron to cells throughout the body.²⁹⁻³⁰ In iron deficiency, the ferritin concentration decreases, indicating reduced iron stores in the body, while the transferrin level increases, reflecting the condition where the body tends to capture more iron from circulation.³¹ Treatment with KKD reversed the situation by normalizing the levels of ferritin and transferrin, indicating recovery from IDA. One of the limitations of this study is that the between-group baseline data comparison for all haematological parameters, except haemoglobin, was not performed to avoid the effect of blood drawing on the anemia.

In this study, the effect of KKD on erythropoiesis was examined by assessing the proportion of erythroid cells at various stages of maturation in the bone marrow. Iron, the central micronutrient, plays a pivotal role in numerous physiological processes, such as Hb synthesis.³² Iron deficiency not only causes a reduction in erythrocyte production but also significantly disrupts erythropoiesis, the process of erythrocyte production, ultimately leading to anemia.³³⁻³⁵ Erythropoiesis is a highly organized and regulated process involving the sequential differentiation and maturation of hematopoietic stem cells into mature RBCs in the bone marrow. In iron deficiency anemia, the reduced availability of iron impairs the proper erythropoietic functioning of the bone marrow, leading to the reduced production of RBC with less Hb.³⁶

Our results showed that KKD significantly increased the erythroid lineage, with Ter119+ cells rising compared to the IDA group, indicating an overall enhancement in the proliferation and differentiation of the erythron, suggesting that KKD promotes complete erythropoiesis. The findings suggest that the active components in Kulekhara may directly stimulate the proliferation and differentiation of erythroid progenitors in the bone marrow, leading to increased production of red blood cells. Ter119, the erythroid lineage-specific marker expressed from the pro-erythroblast stage to mature erythrocytes, plays a role in regulating erythroid cell development. The increase in the percentage of Ter119-positive cells in the Kulekhara-treated group indicates an expansion of the erythroid cell population, suggesting enhanced erythropoiesis.³⁷ CD71, also known as the transferrin receptor, is highly expressed on pro-erythroblast cells, while it is sequentially reduced and minimized on mature RBCs³⁸. Based on the expression of Ter119 and CD71, the erythroid progenitor cells were classified into EryA, EryB, and EryC. It was observed that iron deficiency impaired the normal progression of EryA, EryB, and EryC differentiation, but KKD restored and improved erythropoiesis. This is the first report to study the erythropoietic effect of Kulekhara. This result was similar to the erythropoietic effects of iron supplements.³⁹

Kulekhara is a rich source of a diverse array of phytochemicals, including flavonoids, phenolic compounds, tannins, alkaloids, terpenoids, sterols, carbohydrates, proteins, and fats.⁴⁰ These phytochemicals might be involved in a complex interplay of several bioactive compounds, which may synergistically contribute to the anti-anemic property of Kulekhara. Kulekhara contains triterpenoids, including saponins, betulin, and lupeol, alongside flavonoids such as apigenin, luteolin, gallic acid, quercetin, and ellagic acid, as well as tannins and fatty acids.^{11,41} Many of the phytochemicals have been reported to improve anemia. Myricetin⁴² improves anemia by blocking the hepcidin pathway⁴³ while quercetin improves iron stores by interacting with ferroportin expression.⁴⁴ Ellagic acid, found in Kulekhara in abundance, has been reported to have a therapeutic effect on IDA.⁴⁵ Though the flavonoids inhibit non-heme iron absorption, it was shown that quercetin serves as the substrate for duodenal cytochrome b (Dcytb), which ultimately increases the iron uptake by DMT1.⁴⁶ Flavonoids prevent iron oxidation, maintaining it in a bioavailable form, which ultimately reduces oxidative stress, a common exacerbating contributor in IDA.⁴⁷ An *in-silico* study revealed that Kulekhara phytochemicals showed affinity towards the central cavity of T-state hemoglobin, serving as an erythropoiesis-inducing agent and ultimately aiding in the management of anemia.⁴⁸ Various mechanisms may individually or synergistically contribute to the anti-anemia effect of Kulekhara, which warrants a detailed study. Our work is limited to the scientific validation of the anti-anemia effect of the ethnomedicinal preparation of Kulekhara. Still, in the future, a detailed study of the contribution of different phytochemicals may help in formulating an improved Kulekhara preparation.

This study concludes that KKD preparation, traditionally used in many households to treat anemia, can be as effective as, or even more effective than, iron supplements. Therefore, it can continue to be considered a beneficial ethnomedicine.

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PEER-REVIEWED CERTIFICATION

During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.