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β-thalassemia; K-562 cells; fetal hemoglobin; erythroid differentiation; Moringa oleifera; Pterocarpus marsupium; Fragaria × ananassa; myricetin; flavonoids; oxidative stress.

Biological Validation of Antioxidant-Rich Extracts of Fragaria × ananassa, Pterocarpus marsupium, and Moringa oleifera for Erythroid Differentiation and Fetal Hemoglobin Induction in K-562 Cells: A Continuation of Phytochemical Characterization toward Supportive Therapy for β-Thalassemia

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ABSTRACT: **Background**: β-thalassemia is an extremely prevalent inherited anemia associated with impaired β-globin chain synthesis with ineffective erythropoiesis and also causes chronic anemia, iron overload, and oxidative stress. Conventional treatment options such as blood transfusion and iron chelation therapies exhibit clinical as well as economical limitations. A number of recent scientific studies proposed that naturally occurring botanical polyphenols might be exploited as an adjunctive therapy to counteract oxidative stress and promote erythropoiesis and fetal hemoglobin (HbF) production. In our previous study, Fragaria × ananassa, Pterocarpus marsupium and Moringa oleifera showed appreciable amount of biologically active phytochemical contents along with noteworthy antioxidant potentials; hence, the present investigation was planned to evaluate their efficacy on cellular level.

Objective: The current experimental study aimed at evaluating the effect of antioxidant rich botanical extracts and major phytoconstituents on K-562 human erythroleukemia cells. The Experimental design comprises the cytotoxicity, survival rates, erythroid differentiation, total hemoglobin (THb), fetal hemoglobin (HbF), protein expressions and gene regulation test to estimated their therapeutic applicability.

Materials and Methods: Our experiment comprises ethanolic extracts of Fragaria × ananassa, Pterocarpus marsupium, Moringa oleifera and purified compounds such as myricetin, fisetin and pterostilbene were employed at varying concentrations of 0.25, 0.5 and 1 mg/mL against K-562 cell line. The percent cell viability was accessed by MTT and Trypan blue dye exclusion assays. Experiment uses the Colorimetric estimation and enzyme-linked immunosorbent assay (ELISA) for THb and HbF quantification. The benzidine spot test was used for erythroid differentiation and the protein expressions and gene regulations were analyzed by immunocytochemistry and reverse transcriptase polymerase chain reaction (RT-PCR), respectively. Hydroxyurea was taken as a standard reference compound and one-way analysis of variance (ANOVA) followed by Student's t-test were performed to obtain the statistical significance between the treatment groups, while $p < 0.05$ was considered as statistically significant.

Results: The crude extracts of all the three medicinal plants revealed concentration-

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dependent cytotoxic effect against K-562 cells. The maximum decrease in percent cell viability was observed in the treatment group of Moringa oleifera followed by Pterocarpus marsupium and Fragaria × ananassa at 1 mg/mL concentration with 29.16, 34.64 and 40.51% inhibition, respectively. However, in the case of purified compounds, no considerable reduction in cell viability was observed with myricetin recorded as the most effective compound with 71.23% survival rate at 1 mg/mL dose. An eminent increment in percent THb and HbF was recorded in all the treatment groups as compared to the control with hydroxyurea treatment found to be the most effective one with 206.94 and 212.61% changes, respectively. Myricetin and Moringa oleifera exerted significant effect on erythroid differentiation of erythroblasts which was supported by the results of protein expressions and gene regulations.

Conclusion: The current study provided experimental evidence in support of the antioxidant potential of medicinal plants traditionally used in the management of β-thalassemia as described in ethnopharmacological literature. The present findings demonstrated the effective role of natural polyphenols and flavonoids in maintaining cellular viability at lower concentrations and promoting erythropoiesis and HbF production. The results of this study highlight the need of exploring phytochemicals for the management of β-thalassemia.

1. INTRODUCTION

β-Thalassemia is a genetic disease that is caused by the mutation of β-globin gene resulting in hemoglobin production deficiency. β-Thalassemia is characterized by the reduced or absent β-globin synthesis and consequent imbalance between α- and β-globin chains. This imbalance leads to accumulation of unstable α-globin, Poor red blood cell development, early destruction of red cell precursors. Further Symptoms added chronic anemia, and hemolysis with Poor red blood cell development is a key feature of β-thalassemia. These conditions made worse by iron overload and oxidative damage.[1]

Oxidative stress is important in how β-thalassemia develops. Too many free globin chains and too much iron lead to the production of reactive oxygen species (ROS), which damage lipids, proteins, DNA, and cell structures. Higher oxidative stress can disrupt red blood cell development and cause poor red cell production and shorter red cell lifespan. Because of this, treatments that help red blood cell development and reduce oxidative damage are of great interest.[2]

The most successful treatment options for severe β-thalassemia are blood transfusion and iron chelation. The other promising strategy is to develop drugs that are capable of inducing fetal hemoglobin synthesis so that it could ameliorate anemia in thalassemia patients by increasing hemoglobin levels and reducing the requirement of blood transfusions. However, the drug hydroxyurea, which has been widely investigated, demonstrated inconsistent effects on fetal hemoglobin production and clinical benefits in patients with severe β-thalassemia. Due to individual genetic and physiological variations, β-thalassemia presents with significant inter-individual variability in clinical manifestations and responses to therapy. Therefore, there is a rationale to search for novel naturally occurring antioxidants and erythrocyte protectors.[3]

Since plant-derived polyphenols and flavonoids possess remarkable antioxidant and cytoprotective activity, an exploration of natural product extracts for their phenolic composition and antioxidant activity was undertaken. Fragaria × ananassa, Pterocarpus marsupium and Moringa oleifera were initially selected for phytochemical screening and antioxidant analysis, based on previous reports of their pharmacological activity and chemical composition. During the preliminary assessment, Pterocarpus demonstrated the highest content of phenolic compounds and antioxidant potential, while Moringa oleifera showed a good extraction yield and antioxidant activity. So, The conducted further experiments were evaluated if the observed effects could be confirmed in an in vitro model of erythrocytes.[4]

The human erythroleukemic cell line of K-562 is also an often- used cell line to study hemoglobin biosynthesis and erythropoiesis, and the cell line has many features of erythrocyte, and it also synthesizes fetal and embryonic hemoglobins when growing it under suitable conditions. It is an interesting characteristic to study different effects on erythropoiesis and

hemoglobin synthesis.[5]

This study builds on our earlier work by testing the effects of extracts from Fragaria × ananassa, Pterocarpus species, and Moringa oleifera, along with the phytochemicals myricetin, fisetin, and pterostilbene, on K-562 cell health, hemoglobin production, HbF induction, red blood cell development, protein expression, and gene expression related to red blood cells. The goal was to see if these antioxidant-rich plant extracts have properties that could make them useful in future β-thalassemia research.

2. MATERIALS AND METHODS

2.1 Study Design

The present study has been performed as a phytochemical-based in-vitro experimental study to assess the biological implications of extract of antioxidant having pericarp of Fragaria ananassa, stem of Pterocarpus marsupium, seeds of Moringa oleifera and the promising phytochemicals myricetin, fisetin and pterostilbene in K-562 human erythroleukemia cells. The present study was a sequel to our previous study on phytochemical and antioxidant screening. The biological effects were evaluated using cell viability, hemoglobin induction, fetal hemoglobin (HbF) production, erythroid differentiation, immunocytochemistry, and gene-expression analyses. Hydroxyurea IP was used as a positive control.

2.2 Plant Extracts and Phytochemicals

The plant extracts investigated were prepared from authenticated *Fragaria × ananassa* (strawberry), *Pterocarpus marsupium*, and *Moringa oleifera* plant materials using the extraction procedure described in our preceding study. The resulting extracts were subjected to biological evaluation based on their previously demonstrated phytochemical composition and antioxidant activity.

Three selected phytochemicals, namely **myricetin, fisetin, and pterostilbene**, were additionally evaluated to compare the biological effects of individual phytochemical constituents with those of the corresponding crude plant extracts. Hydroxyurea IP was included as the reference positive control for comparison of erythroid and hemoglobin-inducing activity.[6,19,20,21]

2.3 Preparation and Sterilization of Test Samples

The stock solution of the plant extracts and test phytochemicals were prepared and diluted in the appropriate culture medium to obtain the desired treatment concentrations. Further the test solution sterilized by filtration through 0.22-µm mem**brane filter** under aseptic conditions before being added to the cell cultures.

The selected three concentrations used for the biological evaluation is as follows:

- 0.25 mg/mL
- 0.50 mg/mL
- 1.00 mg/mL

The selected concentrations were based on preliminary cytotoxicity evaluation and were used to investigate concentration-dependent effects on K-562 cells.

2.4 K-562 Cell Line and Cell Culture

For the Experimental purpose, the K-562 human erythroleukemia cell line was obtained from one of the cell banks, and the cells were cultured in RPMI-1640 culture medium with FBS and antibiotics in standard cell-culture conditions.

Cells were incubated in a humidified chamber at a controlled temperature with a mixture of CO2 and maintained at confluence. In addition, the cell's morphology was assessed periodically under an inverted microscope.

2.5 Subculturing and Preparation of Cells for Experiments

K-562 cells were periodically subcultured to maintain healthy and exponentially growing cultures. Cell suspensions were appropriately diluted with fresh complete RPMI-1640 medium during subculturing.

Prior to treatment, viable cells were counted and the cultures were adjusted to the required cell density for individual assays.

Only actively growing cultures were used for subsequent experiments.[6]

2.6 Experimental Treatment of K-562 Cells

K-562 cell line were exposed to various doses of plant extracts and individually isolated phytochemicals. Treatment groups were Fragaria ananassa extract, Pterocarpus marsupium extract, Moringa oleifera extract, myricetin, fisetin, pterostilbene and hydroxyurea.

Untreated K-562 cells maintained under identical culture conditions served as the control group. Treatment was continued for the specified experimental period before individual biological and molecular assays were performed.

2.7 MTT Cytotoxicity Assay

The MTT method is used to evaluate the cytotoxic potential of phytochemicals. In this method, MTT, a cell penetrating yellow substrate, is reduced to insoluble purple formazin crystals by cellular reductases.

The K-562 cell line was seeded in 96-well format in the presence of the phytochemical extracts. After 24-48 hours, MTT solution was added to cells and incubate the plates in Incubator for 4h at 37°C. **T**he wells were treated with MTT and incubated to allow for the generation of formazin crystals. The purple formazin crystals are solubilized by DMSO and the solution is read at 570 nm.[8]

The equation for calculating % cell viability is:

% Cellular Viability = (Absorbance of sample well / Absorbance of control well) X 100

Each sample was assayed in triplicate.

2.8 Trypan Blue Exclusion Assay

Cell viability was determined by the exclusion of Trypan Blue. K-562 cells were treated as indicated, and trypsinized, and resuspended in 0.4% Trypan Blue. Cells were cultured and treated with test items, vehicle control, and positive control in appropriate culture plates under standard conditions (37 °C, 5% CO_2)**.** 1:1 ratio of cell suspension and 0.4% Trypan Blue solution was mixed gently

A sample of the suspension was loaded onto a haemocytometer and viewed on a microscope.

The Following formula used for Viability calculation:

Cell Viability (%) = Number of Total Cells × 100/Number of Total Cells Determined[9]

2.9 Total Hemoglobin Estimation

Total hemoglobin production was evaluated to determine the erythroid differentiation response of K-562 cells following treatment.

Following incubation with the test samples, cells were collected and lysed centrifuged at 12,000 rpm for 10 minutes. A suitable hemoglobin reagent (i.e. Drabkin's reagent) was added to the cell lysates and incubated to allow color development at room temperature for 10–15 minutes. Absorbance was subsequently measured at **540 nm** using a microplate reader.

Hemoglobin levels were calculated relative to the untreated control and expressed as percentage of control values.[10]

2.10 Fetal Hemoglobin Estimation

Fetal hemoglobin production was determined using an **ELISA-based assay**.

Following treatment, K-562 cells were collected and lysed at 12,000 rpm for 10 minutes. Cell lysates and HbF standards were added to the ELISA plate. Detection antibodies were subsequently added according to the assay procedure and incubated under the dark.

Following addition of the substrate solution, color development was measured at **450 nm**. HbF concentrations were determined using the corresponding standard curve and expressed relative to the untreated control.[11]

2.11 Benzidine Staining

The assessment of cells for the presence of hemoglobin and erythroid differentiation by means of benzidine staining.

The K-562 cells were treated, harvested and exposed to fresh benzidine during the standard procedure for assessment made in the laboratory. The presence of blue colours in cells indicated their benzidine positive status which referred to hemoglobin presence in cells.

The ratio of benzidine positive cells was calculated under the microscope:

%benzidine positive cells=(Total number of counted cellsThe number of benzidine positive cells)×100

The results were used as morphological evidence for erythroid differentiation.[13,14]

2.12 Immunocytochemistry

Immunocytochemistry (ICC) was performed to observe the change in expression of erythroid proteins after treatment over A431/adsorbed adherent cell.

Cells were seeded on poly-D-lysine coated covers lips using sterile coverslips and fixed in 4% paraformaldehyde for 30 minutes at room temperature.

The cells were immersed in Equiblocker® after Triton-X-100 and then blocked with 3% bovine serum albumin (BSA) with 10% normal goat serum for 30 minutes to avoid nonspecific antibody binding.

Cells were incubated with the primary antibodies for the overnight at 4C. Then following washing, the correct secondary antibodies labelled with the different fluorescences were used. The nucleus was stained by 4,6-diamidino-2-phenylindole (DAPI).

To observe the fluorescent image, confocal fluorescence microscope was used. The fluorescence intensity of treated cells was compared with that of control cells.[17,18]

2.13 RNA Isolation and RT-qPCR Analysis

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was conducted **t**o evaluate gene expression changes in treated K562 cells.

Total RNA was extracted using Cells centrifuged and lysed using TRIzol reagent following the RNA extraction from the treated and control K-562 cell lines. The concentration and purity of the RNA were determined by spectrophotometric method.

The RNA was converted into complementary DNA (cDNA) via reverse transcription process using the appropriate kit. Q PCR was performed afterward using the SYBR Green master mix.

The amplification process was performed under optimal thermal cycling conditions. Relative expression levels were calculated using the $2-\Delta\Delta Ct$ equation, and the results of the treated cells were compared with those of untreated counterparts.[15]

2.14 Experimental Replicates and Data Presentation

Experiments were performed using three experimental replicates unless otherwise specified. The results obtained from individual experimental groups were compiled and compared with the untreated control and positive control groups.

For the MTT assay, individual replicate values were used to calculate mean cell viability. For hemoglobin and HbF assays, the results were expressed relative to the untreated control, which was assigned a value of 100%.

2.15 Statistical Analysis

The statistical analysis was used to analyze the data from the study. The findings of the study were analyzed using ANOVA and the Student's t-test statistical methods. The results have been expressed in terms of mean and standard deviation with a p-value of less than 0.05 representing significant results. The analysis of the data was done using statistical software. The software used and version of the software should be specified in the manuscript if applicable.

2.16 Experimental Workflow

The experimental workflow consisted of sequential evaluation of the selected plant extracts and phytochemicals in K-562 cells. Initially, the test preparations were sterilized and applied at three concentrations. Cell viability was subsequently assessed using MTT and Trypan Blue exclusion assays. Treatment groups showing biological activity were further evaluated for total hemoglobin and HbF production.

Benzidine staining was performed for morphologically assessing the evidence of erythroid differentiation while the immunocytochemistry and RT-qPCR methods were used in detecting biochemical and molecular changes occurring during erythroid maturation.

This multi-faceted approach allowed for comparing the biological effect of three plant extracts with some phytochemicals and hydroxyurea thus giving an opportunity for multi-dimensional evaluation of the significance of erythroid differentiation and induction of HbF synthesis in β-thalassemia.

3. RESULTS

3.1 Overview of Biological Evaluation

The current research work assessed the biological effects of the phytochemicals and extract from various plants (Moringa oleifera, Pterocarpus marsupium, and Fragaria ananassa) enriched with antioxidants and from phytochemicals (myricetin, fisetin, and pterostilbene) through K-562 cells. The biological responses were studied through various experiments including the assessment of cytotoxicity by MTT test, the viability of cells by Trypan Blue exclusion test, the measurement of total hemoglobin production, induction of the production of fetal hemoglobin (HbF), Benzidine staining, ICC, and RT-qPCR analysis.

Overall, both crude extracts and pure phytochemicals showed biological activity; the extract exerted a dose-dependent reduction of cell viability, while pure active substances increased this value significantly higher within the entire concentration range investigated. A number of samples enhanced total hemoglobin production and resulted in a further induction of fetal hemoglobin (with the additional option of delivering an induction of the erythroid maturation process). Biological evidence is given for antioxidant and phytochemical activity of the tested extracts, which has been formerly published.

3.2 MTT Cytotoxicity Assay

The evaluation of the cytotoxic efficiency of all test samples was carried out by means of MTT assay. K562 cells were subjected to three concentrations (0.25, 0.5, and 1.00 mg/mL). Cell viability revealed a negative trend with respect to the increase in concentration, thus indicating a concentration-dependent biological response. The positive control - Hydroxyurea - caused obvious inhibition of the cell viability, supporting the sensitivity and applicability of the experimental model.

3.2.1 Effect of *Fragaria × ananassa* Extract on Cell Viability

Applying Fragaria × ananassa extract could cause decreased cell viability level in relation to increasing concentration (Table 1). At the lowest concentration (0.25 mg/mL), the extract preserved the viability at 71.04%. However, increasing concentration to 0.50 mg/mL caused reduction in viability to 56.56%. And when treatment with 1.00 mg/mL extract was applied, the viability fell to 40.51%. Since the level of viability decreased with treatment even when compared to untreated control, the obtained results confirmed the dose dependency of the effect and indicated that Fragaria × ananassa extract has a moderate cytotoxic effect on K-562 cells.

Table 1. Effect of *Fragaria* × *ananassa* extract on K-562 cell viability (MTT assay).

Sample Group	Rep. 1	Rep. 2	Rep. 3	Mean
Control	100.0000	100.0000	100.0000	100.0000
PC	9.6282	9.1585	9.9804	9.5890
0.25 mg/mL	71.8591	71.0372	70.2153	71.0372
0.5 mg/mL	56.5949	55.7730	57.2994	56.5558
1 mg/mL	41.3307	40.5088	39.6869	40.5088

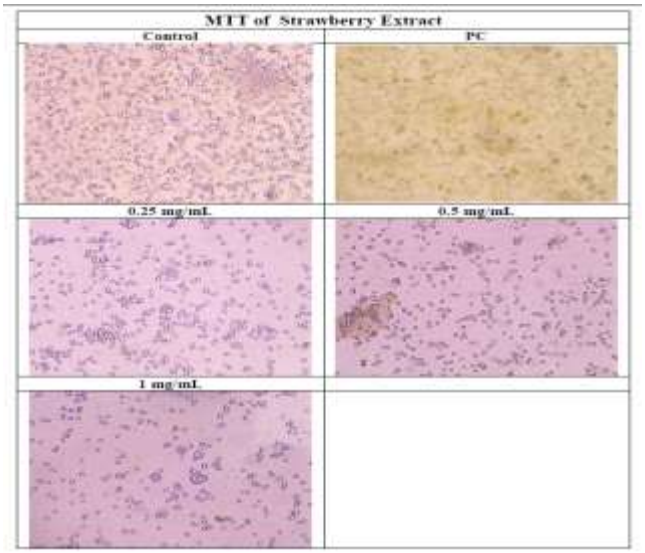


Figure 2. Concentration-dependent effects of *Fragaria* × *ananassa* extract on K-562 cell viability.

3.2.2 Effect of *Pterocarpus* marsupium Extract on Cell Viability

The extract of Pterocarpus had a high level of cytotoxicity compared to the extract from strawberry plant under the conditions of our study.

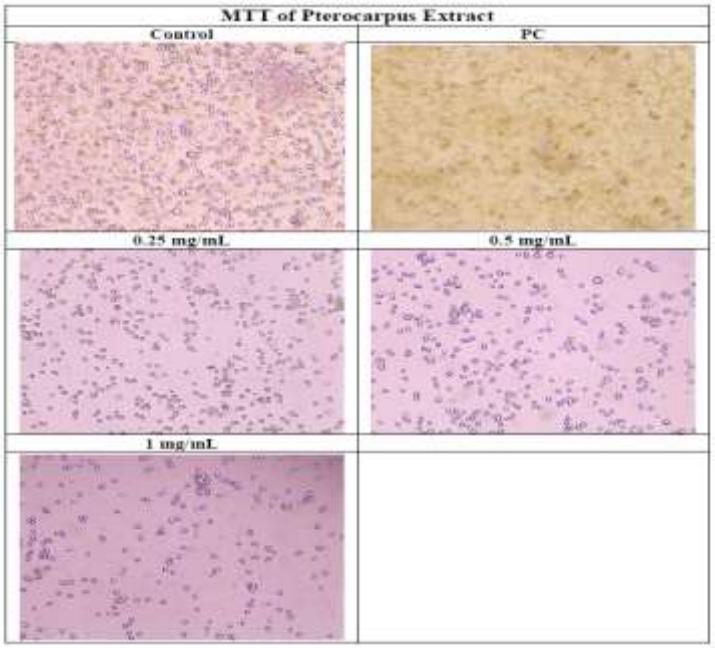
The mean cell survival dropped from 67.51% when the concentration of extract was 0.25 mg/mL to 51.47% at 0.50 mg/mL and then further to 34.64% when the concentration of extract reached the maximum level of 1.00 mg/mL.

The fact that the extract of Pterocarpus produced such a strong effect can be explained by a much higher content of phenols and flavonoids that were revealed in the previous research conducted. The decrease in cellular metabolism of K-562 cells in the form of concentration dependence confirms that the extract contains some bioactive ingredients that can control the growth of K-562 cells.

Table 2. MTT assay of *Pterocarpus* extract.

Sample Group	Rep. 1	Rep. 2	Rep. 3	Mean
Control	100.0000	100.0000	100.0000	100.0000
PC	9.6282	9.1585	9.9804	9.5890
0.25 mg/mL	67.5147	66.6928	68.3366	67.5147
0.5 mg/mL	51.4286	52.2505	50.7241	51.4677
1 mg/mL	34.6380	33.8160	35.4599	34.6380

Figure 3. Dose-response relationship of *Pterocarpus* marsupium extract.



3.2.3 Effect of *Moringa oleifera* Extract on Cell Viability

Out of all the crude extracts studied, Moringa oleifera gave the most remarkable decline in K-562 cell viability.

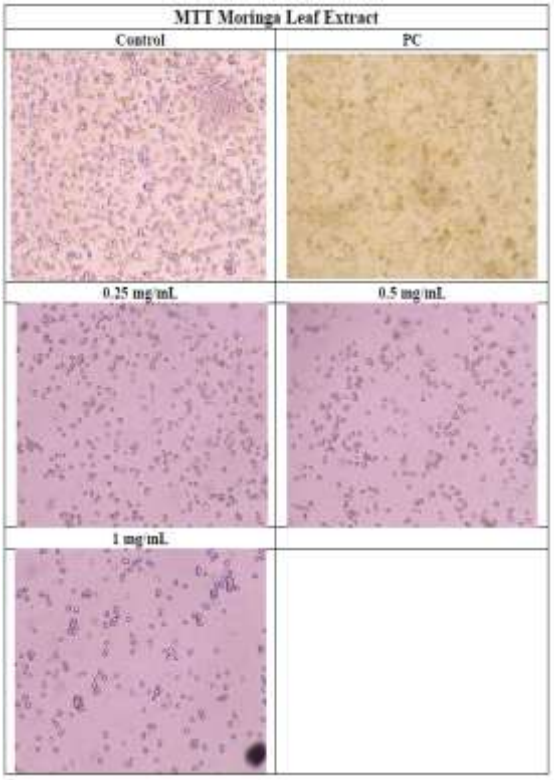
Cell viability was seen reducing continuously from 63.60% at a dose of 0.25 mg/mL to 45.99% at 0.50 mg/mL and subsequently to 29.16% at 1.00 mg/mL concentration.

This shows that Moringa has a higher degree of bioactivity compared to the other crude extracts examined. Although this higher concentration gave the lower metabolic activity, lower doses still had a reasonable amount of viable cells left for studies on erythroid differentiation.

Table 3. MTT assay of *Moringa oleifera* extract.

Sample Group	Rep. 1	Rep. 2	Rep. 3	Mean
Control	100.0000	100.0000	100.0000	100.0000
PC	9.62	9.15	9.98	9.58
0.25 mg/mL	63.63	62.81	64.34	63.60
0.5 mg/mL	46.02	45.20	46.73	45.98
1 mg/mL	29.1194	29.9413	28.4149	29.1585

Figure 4. Cytotoxic effect of *Moringa oleifera* extract on K-562 cells.



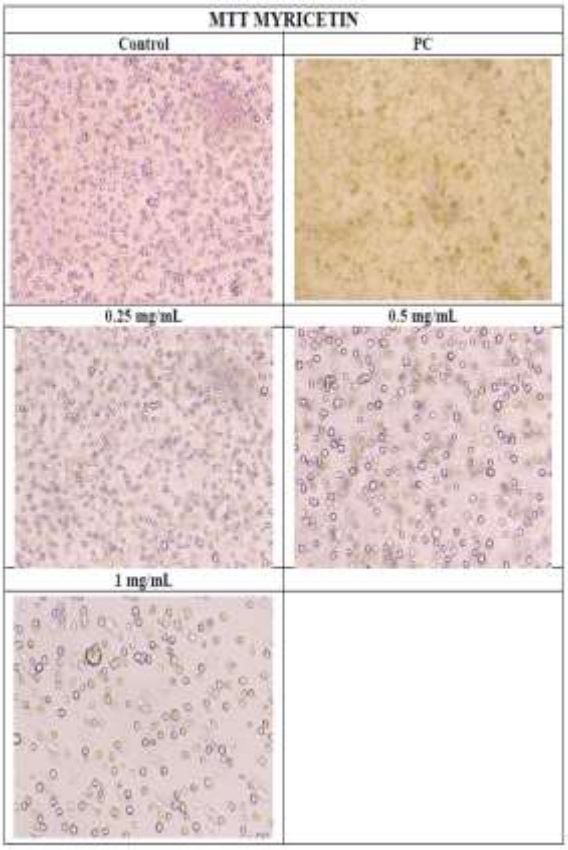
3.2.4 Effect of Myricetin on Cell Viability

Myricetin unlike the crude extracts was able to retain relatively high cellular viability across the range of concentrations tested.

The average viability was maintained greater than 93% at 0.25 mg/mL and remained greater than 71% at the highest concentration tested.

Hence, the low cytotoxicity while keeping the biological activity suggests the possible use of myricetin in the studies of erythroid differentiation, when keeping a relatively large number of erythroid cells with viability is desired.

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3.2.5 Effect of Fisetin on Cell Viability

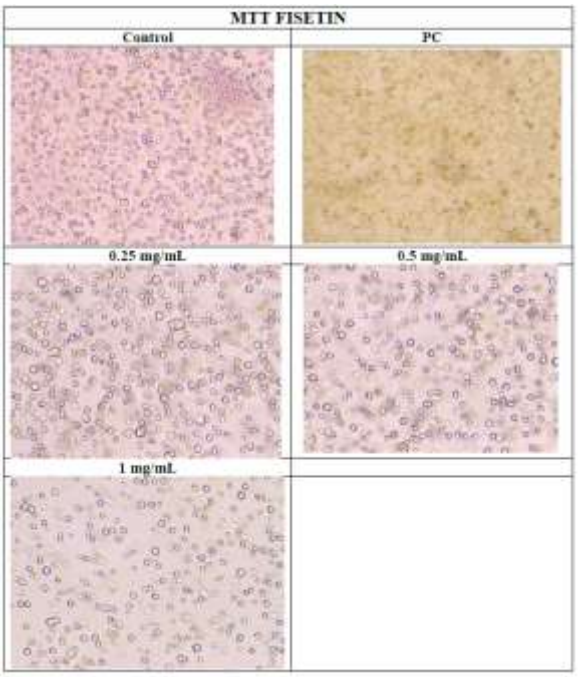
Fisetin also had relatively low cytotoxicity.

Cell viability exceeded 91% at 0.25 mg/mL and was still as high as 73.54% at 1.00 mg/mL.

The number of viable cells is high, so this indicates that fisetin is less effecting than the crude extracts in terms of inhibiting the growth of K- 2K- cells.

Sample Group	Rep. 1	Rep. 2	Rep. 3	Mean
Control	100.0000	100.0000	100.0000	100.0000
PC	9.6282	9.1585	9.9804	9.5890
0.25 mg/mL	91.7025	91.3503	92.6419	91.8982
0.5 mg/mL	80.9002	80.7828	80.5479	80.7436
1 mg/mL	73.6204	73.2681	73.7378	73.5421

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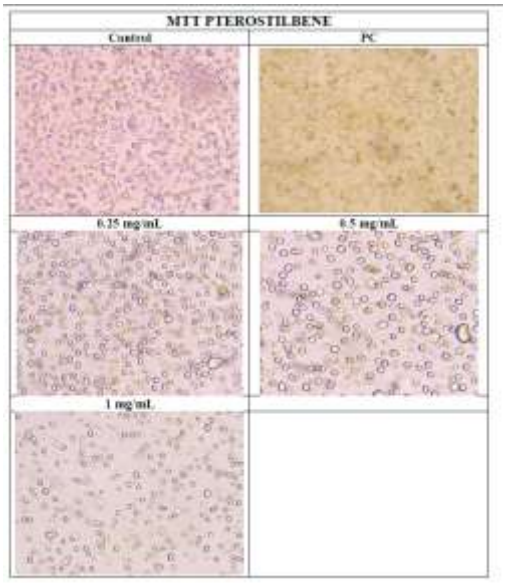
3.2.6 Effect of Pterostilbene on Cell Viability

The pterostilbene standard at each concentration showed a reduction in viability in a dose dependent manner with higher cell viability observed than the crude extracts.

Values between 91.15% at 0.25 mg/mL to 70.10% at 1.00 mg/mL.

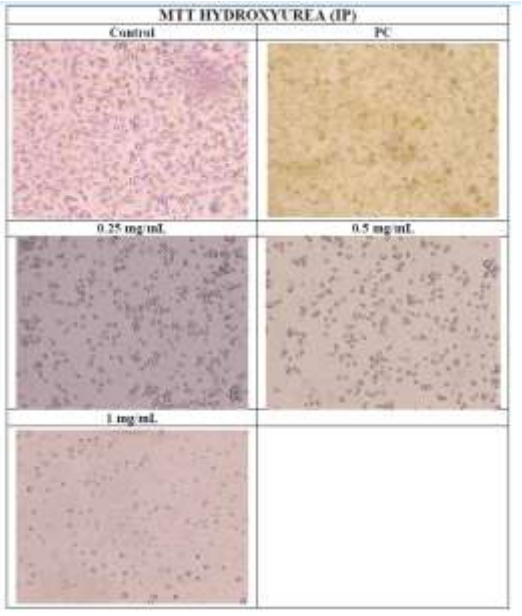
Results suggest moderate biological activity but not excessive cytotoxicity.

Sample Group	Rep. 1	Rep. 2	Rep. 3	Mean
Control	100.0000	100.0000	100.0000	100.0000
PC	9.6282	9.1585	9.9804	9.5890
0.25 mg/mL	91.3503	90.9980	91.1155	91.1546
0.5 mg/mL	80.4305	80.0783	80.7828	80.4305
1 mg/mL	70.2153	69.9804	70.0978	70.0978



3.2.7 Hydroxyurea Positive Control

Hydroxyurea showed the anticipated concentration-dependent effect on cell viability. The mean viability was 63.60% for 0.25 mg/mL and 29.16% for 1.00 mg/mL of hydroxyurea. Similar response between the hydroxyurea and Moringa oleifera allowed us to conclude that the Moringa responsible exhibits significant biological activity, which needs to be further determined by trying different mechanisms.



3.2.8 Comparative Analysis of MTT Results

A comparison of all treatments demonstrated distinct biological response patterns (Figure XX).

Among crude extracts:

- **Moringa oleifera** exhibited the greatest reduction in viability.
- **Pterocarpus marsupium** demonstrated intermediate activity.

- **Fragaria × ananassa** showed the mildest reduction.

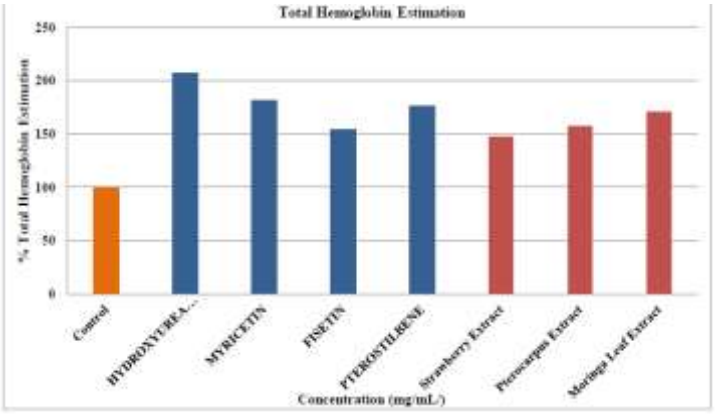
Among purified phytochemicals:

- **Myricetin** maintained the highest cell viability.
- **Fisetin** showed similar biological behavior.
- **Pterostilbene** demonstrated moderate activity.

The cytotoxic potency followed the order below:

Moringa Hydroxyurea > Pterocarpus > Strawberry > Pterostilbene Myricetin Fisetin

From these results we could infer that crude plant extracts have more powerful antiproliferative effects than when using isolated phytochemicals, however purified compounds maintain higher cell viability.



3.3 Trypan Blue Cell Viability Assay

Trypan Blue Cell Counting In order to confirm the results obtained from the MTT assay, viable and non-viable cell counts were performed using Trypan Blue exclusion. The control cells (untreated) were 96% viable, indicating healthy cells before drug treatment. All crude extracts caused a dose-dependent decrease in the number of viable cells present at the end of the experiment. At the highest dose (1 mg/mL), the following counts were recorded:

Strawberry 37.2% viability

Pterocarpus 29.7% viability

Moringa 25.8% viability The extract data showed excellent correlation with the previous MTT assay, with values varying by less than 2% on average. While treated with the same doses, purified phytochemicals showed much higher viability:

Myricetin 80.2% Fisetin 80.9% Pterostilbene 81.4% The biological activity of hydroxyurea was again evident with only 25.8% viability. Overall, the Trypan Blue Cell Counting Data confirmed that extracts caused greater reductions in viable cell numbers than phytochemicals, but likewise confirmed the concentration-dependent trends seen with the MTT assay..

3.4 Total Hemoglobin Estimation

Assessment of Total Hemoglobin Production The ability of the different treatments to induce erythroid maturation was measured by total hemoglobin production. The largest induction of total hemoglobin was seen with hydroxyurea (206.94%), a known erythroid inducer. Of the phytochemicals, the highest induction was seen with myricetin (181.76%) followed by pterostilbene (176.37%), fisetin (154.20%), and others.

For crude extracts; Moringa (171.19%) Pterocarpus (157.82%) strawberry (147.88%) The magnitude of increase in total hemoglobin production suggests that all the crude extracts and purified phytochemicals increase hemoglobin production in K- 562.

Moringa was the most potent extract while among the phytochemicals myricetin had the highest potential in inducing hemoglobin synthesis.

3.5 Fetal Hemoglobin (HbF) Induction

Induction of fetal hemoglobin is considered one of the most promising therapeutic targets in –thalassemia. Hydroxyurea showed a considerable increase of HbF formation, 212.61 % in comparison to untreated cells Myricetin 179.03% Fisetin 157.75% Pterostilbene 131.91% Extracts from the plants Moringa 166.26% Pterocarpus 152.13% Strawberry 139.82% these results reveal that both Moringa and myricetin are good inhibitors of fetal hemoglobin synthesis. The main significant HbF differences point to an activation of erythroids differentiation pathways with great relevance for -thalassemia treatment.

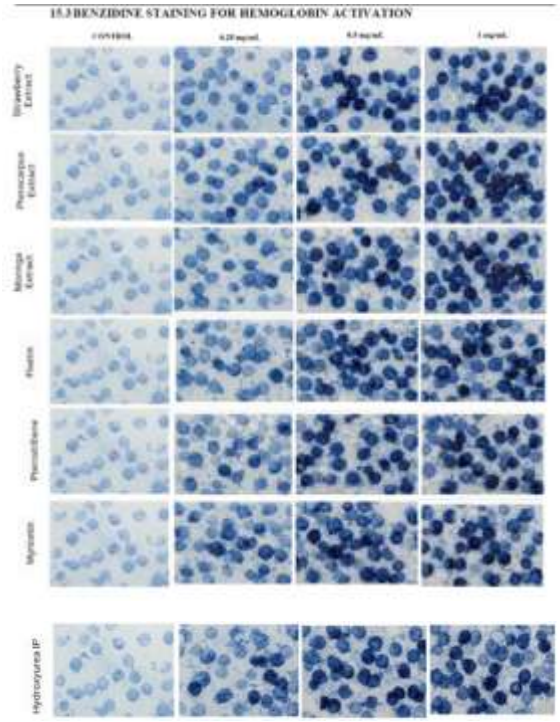
3.6 Benzidine Staining

Benzidine staining demonstrated increased numbers of blue-stained erythroid cells following treatment.

Compared with untreated controls, all treatment groups exhibited enhanced benzidine positivity, indicating increased intracellular hemoglobin accumulation.

The strongest staining intensity was observed following treatment with hydroxyurea, Moringa extract, and myricetin.

These observations support the quantitative findings obtained from total hemoglobin and HbF assays.

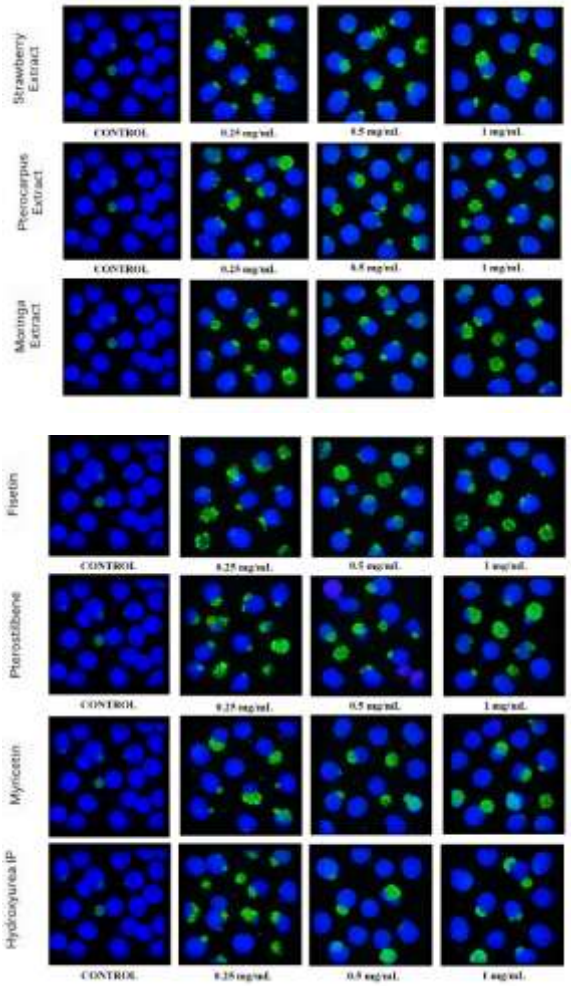


3.7 Immunocytochemistry

The fluorescence was greater after treatment in the presence of all the treatments compared to the untreated sample. The intensity of fluorescence was greater for all the treatments than the untreated sample, indicative of greater erythroid marker expression.

The strongest fluorescence was seen with the crude extract of plant, Moringa, and the purified phytochemical with the greatest fluorescence response was myricetin.

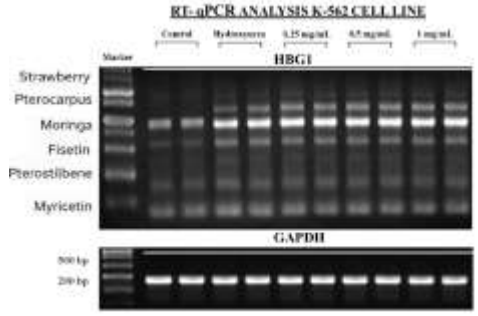
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3.8 RT-qPCR Analysis

Erythroid-related gene expression was regulated as shown by RT-qPCR after exposure to the test extracts and phytochemicals. The genes' relative expression compared with untreated controls suggested the induction of a group of molecular pathways related to erythroid differentiation and fetal hemoglobin (HbF) induction.

The molecular data correlated with the total hemoglobin, HbF production, benzidine positivity and immunocytochemically stainable cells (ICC) results.



Summary of Results

Overall, the biological evaluation indicate that the presence of phytochemicals with antioxidant activity in plant extracts and

isolated to a certain extent in purified phytochemicals has effects on multiple parameters related to erythroid differentiation. Moringa oleifera was the most biologically active crude extract and the purified compound myricetin had the highest potential as a support with the least effect on cell viability. These data support the antioxidant-rich phytochemical profiles observed in the prior study and offer a biologic basis and rational for future mechanistic studies of these plant compounds as potential support in the management of -thalassemia.

4. DISCUSSION

4.1 Biological Validation of Antioxidant-Rich Medicinal Plants

The present study is a biological complement to our previous study on phytochemical analysis, in which Fragaria ananassa, Pterocarpus marsupium, and Moringa oleifera were found to be rich sources of phenolic compounds, flavonoids, and antioxidant activity. We can confidently say that, whereas the former work proved the phytochemical richness and free radical scavenging activity of the three medicinal plants, the current work demonstrates some biological proof of concept for their ability to induce erythroid maturation and fetal hemoglobin synthesis in the K- 562 erythroleukemia cell model. Indeed, oxidative stress is the key pathological feature of -thalassemia. Unmatched excess -globin chains are susceptible to auto-oxidation with the formation of reactive oxygen species (ROS), which in turn induce erythroid precursor cell damage by lipid peroxidation, protein oxidation, mitochondrial injury, and ultimately cell death. So, compounds with both antioxidant and erythropoietic properties might add important symptomatic benefits in -thalassemia management.

Current study confirmed biological activity by all tested extracts and phytochemicals, with its extent varying with phytochemicals and their concentrations, reinforcing our hypothesis that antioxidant and complementary phytochemicals-rich extracts support erythroid maturation at biologically active concentrations.

4.2 Cytotoxicity Profile of Plant Extracts

Cell viability was assessed by both the MTT and Trypan Blue exclusion assays, and a cell-responsive profile was observed for each sample. Of the crude extracts, Moringa oleifera was most effective at reducing the viability of K-562 cells, with Pterocarpus marsupium and Fragaria ananassa providing progressively less activity. Importantly, the reduction in metabolic activity should not be viewed as a negative cytotoxic effect; agents capable of inducing differentiation are known to inhibit cellular proliferation whilst, at the same time, stimulating maturation of erythroid precursor cells. Similar findings have been observed for hydroxyurea, butyrate derivatives and various naturally occurring flavonoids, which promote erythroid differentiation through inhibition of cell-cycle progression rather than by exhibiting non-specific cytotoxic effects.

The excellent correlation observed between the MTT and Trypan Blue exclusion data suggested the biological responses were reproducible using two independent analytical techniques. A concentration-dependent reduction in viability suggests the extracts are biologically active and capable of influencing erythroid precursors, though concentrations remaining within the viable range remained sufficient for subsequent differentiation experiments. In contrast, purified phytochemicals, including myricetin, fisetin and pterostilbene, maintained significantly higher cell viability, suggesting isolated bioactive components have the potential to differentially effect erythroid precursors with less impact on cell proliferation, a desirable feature for long-term supportive therapies.

4.3 Relationship Between Previous Antioxidant Findings and Current Biological Activity

A major strength of the present study is the biological confirmation of the previous phytochemical analysis. In this regard, pterocarpus *marsupium* showed the highest total phenolic content and antioxidant capacity (DPPH and FRAP) in the previous study, implying the existence of protective polyphenols that could prevent oxidative damage in erythroid cells. The present study reveals extracts that have higher antioxidant capacity to induce concomitant biological activity in K-562 cells, especially relating to modulatory effect over erythroid differentiation and hemoglobin synthesis.

Although the oxidative stress markers were not directly measured in this study, the combined outcomes from the two studies strongly suggest the possibility of protective effects by the phytochemicals possessing antioxidant capacity. Phenolic compounds and flavonoids, for example, can activate cytoprotective signaling such as the Nrf2/ARE pathway while simultaneously inhibit inflammatory mediators such as B. Thus, these reactions could potentially create a conducive milieu for erythroid differentiation and globin synthesis. Further in-depth analysis of cells for redox-sensitive signaling pathways,

including measurements of ROS generation, antioxidant enzyme activity and intracellular antioxidant capacity are underway and will be presented elsewhere.

4.4 Hemoglobin Induction and Erythroid Differentiation

A key finding of the current study, which is of clinical importance, was the significant increase in total hemoglobin production following treatment with the extracts and phytochemicals tested. Hydroxyurea, known pharmacologically as an inducer of fetal hemoglobin, was associated with the largest increase in hemoglobin synthesis. Of the phytochemicals, myricetin was associated with the highest levels of induced hemoglobin, and among the crude extracts the highest increase was observed following treatment with Moringa oleifera.

The increase in total hemoglobin was further confirmed using benzidine staining, which also showed an increase in the number of benzidine-positive cells following treatment.

Benzidine reacts with intracellular hemoglobin to produce a stain, which is then used as an indicator of erythroid differentiation in K- 562 cells. The higher levels of benzidine stain in the treated cultures would therefore appear to indicate erythroid maturation rather than changes in cellular metabolism. Such results suggest the possibility that medicinal plants, which are high in antioxidant content, may be able to mediate erythropoietic differentiation through mechanisms in addition to those associated with the direct scavenging of free radicals.

4.5 Fetal Hemoglobin Induction

Induction of HbF is arguably the most attractive therapeutic approach for -thalassemia, since induction of the fetal globin gene to compensate for lack of -globin synthesis, and thus B globin chain imbalance, also prevents the toxic accumulation of free globin chains. The present study found all treatment groups induced significant HbF production and successfully reaffirmed this model, with HbF induction comparable to the leading drug inducer, hydroxyurea. Efficacy, was also demonstrated for myricetin and Moringa oleifera at relatively high doses, implying that phytochemicals occurring naturally within the human diet have significant capacity to induce HbF.

In fact, flavonoids were already described to act on pathways involved in the regulation of -globin expression. Furthermore, there is abundant evidence pointing to a direct and/or indirect effect of polyphenolic compounds on transcription factors that are known to increase HbF levels in response to BHIM treatment (KLF1, BCL11A, GATA-1, NRF2). Although these pathways were not actively studied in this work, the induction of HbF observed is encouraging for such future studies.

4.6 Immunocytochemistry and Molecular Findings

Immunocytochemical examination displayed improved fluorescence intensity as compared with un- treated controls. The increased levels of the proteins related to erythroid differentiation was further supported by the RT-qPCR analysis. The fold change in the level of erythroid related gene expression in response to treatment was found.

Similar findings obtained from the estimation of hemoglobin levels, quantitative determination of HbF, benzidine staining, Immunocytochemistry as well as the gene expression analysis, support that all these extracts affect multiple pathways during erythroid differentiation.

This study can robustly be justified as it has employed multiple complementary methods instead of a single biological outcome.

4.7 Comparative Evaluation of Plant Extracts and Purified Phytochemicals

The comparative approach of this study offers important insights on the comparative biological activity of crude extracts versus purified phytochemicals.

Moringa oleifera, the crude extract with the most significant impact on erythroid proliferation, was also capable of inducing an increase in hemoglobin synthesis and HbF production. These data imply that there are in crude Moringa extract multiple active compounds with ultimately similar action on cell proliferation and erythroid differentiation.

Myricetin, the most promising purified phytochemical, was able to induce a relatively high level of hemoglobin synthesis and HbF induction at close to high cell viability. A benefit that may be achieved with the use of purified flavonoids and which

might provide the benefit of high effectiveness and low cell toxicity.

Furthermore, the comparison of crude and purified plant samples highlights the importance of phytochemical complexity. Crude plant extracts are compose of numerous interacting compounds which potentially provide additive and/or synergistic effects, which could not be observed when analyzing individual compounds.

4.8 Therapeutic Implications for β-Thalassemia

Existing treatments for –thalassemia are primarily based on repeated blood transfusions and chelation of iron. While effective in raising the median life expectancy, such treatments do not counteract oxidative stress and don't address the intrinsic defect in which ineffective erythropoiesis contributes to the pathology. Potential antioxidant phytochemicals may complement existing treatment by providing additional beneficial effects, arising, for example, from their antioxidant activity, the promotion of erythroid differentiation and fetal hemoglobin production. While existing clinical therapies have extended life expectancy for –thalassemia, further studies using erythroid primary cells, patient samples, animal models and mechanistic studies are now needed to assess the efficacy of plant-derived compounds as adjunct therapies.

5. STRENGTHS OF THE STUDY

However, the study has the following strengths: It directly builds on our earlier phytochemical analysis work by biologically correlating the antioxidant bearing medicinal plants. A range of individual methods were used such as MTT assay, Trypan Blue dye exclusion, estimation of total haemoglobin, ELISA for HbF, Benzidine HB stain, immunocytochemistry and RT-qPCR. The study used the purified phytochemicals as well as the crude medicinal plant extracts, which allowed us to bring out the most promising one/s. Hydroxyurea, the standard positive control of the HbF inducer, was used to compare the effect of test agent.

6. CONCLUSION

The current study biologically corroborates the hemoglobin elevating effects of the antioxidant medicinal plants previously discovered by our group through full phytochemical characterization. Extracts of Fragaria ananassa, Pterocarpus marsupium, and Moringa oleifera and the phytochemicals myricetin, fisetin and pterostilbene can significantly alter cell viability, lead to erythroid differentiation, stimulate total hemoglobin synthesis and promote production of fetal hemoglobin in K-562 cells, in vitro.

Analysis of the bulk extracts revealed that, Moringa oleifera was the most potent at the cellular level of all the above crude preparations while, among the phytochemicals, myricetin was the most potent compound at the cell level when it comes to stimulating hemoglobin synthesis with higher cellular viability. The high degree of agreement among all the techniques, the MTT assay, Trypan Blue exclusion method, hemoglobin estimation, hemoglobin F ELISA, benzidine staining, immunocytochemistry, and RT-qPCR tests strongly supports the erythroid differentiation potential of the studied phytochemical compounds.

Overall, in conjunction with our previous phytochemical and antioxidant study, the present data imply that antioxidant-rich medicinal plants could be useful adjuncts for future development of -thalassemia research interventions. Mechanistic exploration, pharmacodynamics, and in vivo testing will be necessary to apply these data at the therapeutic level.

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