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Partial Purification of Glutathione –S- Transferase and Study of Kinetic Properties and Some Antioxidants in People with Leukemia

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ABSTRACT: Glutathione-S-transferase (GST) is a multifunctional detoxification enzyme that plays a central role in maintaining cellular redox homeostasis. Leukemia, a malignant hematological disorder, is often associated with disrupted antioxidant defenses and oxidative stress. During the current work, GST was partially purified from the serum of leukemia patients by employing the ammonium sulfate fractionation (75%), dialysis, and Sephadex G-75 gel filtration. The purification process yielded a final (9.698-fold) increase in purity, exhibiting a specific activity of (1.888 U/mg) and (86.75%) recovery, confirming efficient enrichment of the enzyme. Kinetic evaluation revealed optimum activity at pH 6.25, 0.12 M buffer concentration, and 25 °C, suggesting conformational adaptations of GST under leukemic conditions. Antioxidant status was concurrently assessed by quantifying catalase, reduced glutathione, and total antioxidant capacity (TAC). Leukemia patients exhibited significant reductions in all biomarkers compared with controls: CAT (92.47 ± 12.93 vs 112.78 ± 15.51 mk/L), GSH (10.1 ± 1.33 vs 15.8 ± 2.26 µmol/L), and TAC (196.6 ± 30.81 vs 352.8 ± 52.80 µmol/L). Stratification by disease type indicated that acute leukemia patients had the most profound antioxidant depletion, while chronic patients retained relatively higher levels. Sex- and age-based comparisons revealed additional variability, with females showing lower TAC values than males, and younger ALL patients displaying relatively preserved GSH levels compared with older subtypes. Collectively, these findings demonstrate that GST purification and characterization provide insights into enzyme behavior under leukemic oxidative stress, while the associated antioxidant depletion underscores the potential diagnostic and prognostic value of GST and redox biomarkers in leukemia management.

Keywords: Glutathione-S-Transferase, Leukemia, Partial Purification, Kinetics, Antioxidants, Oxidative Stress

INTRODUCTION

Leukemia represents a heterogeneous group of hematological malignancies characterized by uncontrolled proliferation of abnormal leukocytes and disruption of hematopoiesis. The global incidence of leukemia has been rising, with acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), and chronic myeloid leukemia (CML) representing the major subtypes (Bray et al., 2021). Oxidative stress has been identified as a major hallmark of leukemia progression, where reactive oxygen species (ROS) accumulation disrupts the antioxidant defense system and contributes to DNA damage, apoptosis resistance, and chemotherapy response (Reuter et al., 2019). Glutathione-S-transferases (GST) are multifunctional enzymes that facilitating the conjugation of glutathione (GSH) with a broad range of electrophilic molecules, thereby detoxifying xenobiotics and endogenous metabolites (Hayes & Strange, 2020). Beyond detoxification, GSTs play critical roles in regulating apoptosis, modulating stress-activated signaling, and maintaining redox balance. Alterations in GST expression and activity have been associated with hematological malignancies, and genetic polymorphisms in GST isoenzymes have been linked to increased leukemia susceptibility (Bolt & Thier, 2006; Saadat et al., 2022). Antioxidant biomarkers such as catalase, reduced glutathione, and total antioxidant capacity (TAC) provide insights into the systemic oxidative stress status. Previous studies have demonstrated significant decreases in antioxidant enzymes in leukemia patients, correlating with disease severity and poor prognosis (Khan et al., 2021; Nunes et al., 2022). The current study aimed to (i) partially purify GST from serum of leukemia patients, (ii) determine its kinetic properties under optimal experimental conditions, and (iii) evaluate the antioxidant profile (CAT, GSH, TAC) in relation to clinical and

demographic factors. The results provide a comprehensive biochemical perspective on the role of GST and antioxidant defense in leukemia.

MATERIALS AND METHODS

Sample Collection

One hundred seven (107) samples of serum blood of patient having leukemia cancer of both main types (Chronic &Acute). All of them have taken chemotherapy. Fifty-five sample of serum blood were used as a control group. The age of patients ranged between (9-65) years. Those patients visited the Kirkuk oncology hospital during the period from 10th November 2023 till 15th March 2025. The samples have been classified into Two groups as below: Control group included (65) samples using as healthy people with ages range between (9-65) years. Seconded group include, patients with same age range of first group, have diagnosis with leukemia cancer which taken chemotherapy. For each cancer patient, 3 ml of venous blood was obtained and healthy subjects were rescued by antecubital vein puncture and stressful venipuncture. Samples were immediately transferred into gel tubes, and left at room temperature for 15 minutes to allow it to clot and then centrifuged for 15 minutes at 7000 Xg to ensure complete separation. From blood serum. Serum samples were divided into 3 aliquots and stored frozen at (−20°C) to avoid analysis.

Working Reagents

Total Antioxidant Estimation

The principle depend on the method (Rubio et al., 2016) and explan as followed:

a. Preparation of Acetate buffer (300 mmol/L, pH 3.6):[0.775 g of Sodium acetate trihydrate ($C_2H_9NaO_2.3H_2O$) was dissolved in 4 mL of acetic acid, and the volume was adjusted to 250 mL with distilled water.

b.Preparation of 2,4,6-tripyridyl-S-triazine (TPTZ, 10 mM) solution:[0.0625 g of TPTZ (MW: 312.33) was dissolved in 0.8 mL of 1 M HCl, and the final volume was made up to 20 mL with distilled water].

c. Preparation of $FeCl_3.6H_2O$ solution (20 mM):[0.108 g of FeCl3.6H_2O (MW: 270.33) was dissolved in distilled water, and the final volume was adjusted to 20 mL].

d. Preparation of FRAP working solution:The working solution was freshly prepared by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution (1:10 dilution), and 2.5 mL of FeCl3.6H_2O solution.

e. Preparation of $FeSO_4.7H_2O$ standard solutions:A series of standard $FeSO_4.7H_2O$ solutions (150, 200, 250, 300, 350, 400 mmol/L) were prepared. 0.2780 g of $FeSO_4.7H_2O$ was dissolved in distilled water, and the volume was completed to 100 mL. The working standards were obtained by appropriate dilution. The absorbance of the FRAP reagent was measured at 593 nm.

Working Method

The procedure was performed according to the standard FRAP method. Briefly, 1 mL of the sample was mixed with 2 mL of freshly prepared FRAP reagent. The mixture was incubated for 10 minutes at 37 °C, and the absorbance was recorded at 593 nm. The antioxidant capacity was then calculated using the calibration curve constructed with Fe^{2+} standard solutions.

Estimation of Serum Catalase (CAT) Activity

Phosphate buffer (50 mM, pH 7.0): Prepared by mixing two stock solutions: (a) 6.81 g KH_2PO_4 dissolved in 1000 mL distilled water, and (b) 8.90 g Na2HPO4.2H2O dissolved in 1000 mL distilled water. Solutions (a) and (b) were mixed at a ratio of 1:1.55. Hydrogen peroxide (30 mM): Prepared by diluting 0.34 mL of 30% H_2O_2 with phosphate buffer to a final volume of 100 mL. the principle depends on the (Heck et al., 2003).

Procedure:

Serum samples were freshly diluted by adding 0.010 mL of serum to 1 mL of phosphate buffer. The assay was started by adding 0.5 mL of H_2O_2 (30 mM) to 1 mL of the diluted serum sample and mixing well. Absorbance was recorded at 240 nm after 15 seconds (A1) and again after 30 seconds (A2).

Calculation: Catalase activity (K/mL) was calculated using the following equation:

$$[\text{Catalase K/mL} = (\text{Vt} \backslash \text{Vs}) * (2.3 \backslash \Delta\ \text{T}) * \log (\text{A1} \backslash\ \text{A2}) * 60] \quad (1)$$

Estimation of serum Reduced Glutathione

The reagents required for the estimation of serum reduced glutathione (Elsyade et al., 2021) included sulfosalicylic acid solution (4%), phosphate buffer at pH 8.0, and Ellman's reagent. The phosphate buffer (pH 8.0) was prepared by mixing 0.6 M KH_2PO_4 with 0.08 M K_2HPO_4 and adjusting the final pH to 8.0. Ellman's reagent (0.1 mM) was freshly prepared by dissolving 0.004 g of DTNB in the phosphate buffer (pH 8.0) and stored in a dark bottle to prevent degradation. For the test sample, 150 µL of serum was mixed with 150 µL of 4% sulfosalicylic acid, while in the blank tube, 150 µL of distilled water was used instead of serum but treated with the same volume of sulfosalicylic acid. Both mixtures were vortexed and centrifuged at 3000 ×g for 5 minutes. After centrifugation, 150 µL of the clear supernatant from each tube was transferred into separate clean test tubes. To each tube, 4.5 mL of Ellman's reagent (0.1 mM) was added and mixed thoroughly. The absorbance of the reaction mixture was then measured at 412 nm against the blank. Reduced glutathione concentration was calculated using the following equation:

$$[\text{GSH Conc. (µmol/L)} = (A_{test} - A_{blank} / \varepsilon_{\circ} \times \text{d}) \times 10^6] \quad (2)$$

PURIFICATION OF GLUTATHIONE-S-TRANSFERASE (GST) FROM SERUM

GST enzyme was purified from the serum of a 45-year-old male leukemia patient under cold conditions (4°C). Initially, (7.5) gm of solid ammonium sulfate (75% saturation) was gradually added to 10 mL of serum with continuous stirring for 60 minutes, followed by incubation at 4°C for 24 hours. The mixture was centrifuged at 2775 ×g for 45 minutes, and the resulting protein pellet was dissolved in phosphate buffer (1 mL). Total protein concentration was measured at 550 nm, and GST activity was determined at 340 nm (Habig et al; 1974). The protein fraction with the highest GST activity was subjected to dialysis using a semipermeable membrane (MWCO 8,000–14,000 Da) against 1000 mL phosphate buffer (0.1 M, pH 6.25) at 4°C for 48 hours, with the buffer replaced every 6 hours to remove residual salts. For further purification, 13 g of Sephadex G-75 gel was hydrated and allowed to swell at 4°C for 72 hours. Fine particles were removed, and the gel slurry (~75% of settled matrix) was carefully packed into a 1.5 × 50 cm glass column and equilibrated with phosphate buffer (pH 6.25) for 24 hours. A 9 mL protein sample from dialysis was applied to the top of the column, and elution was performed with phosphate buffer (pH 6.25). Fractions of approximately 3 mL were collected every 1.5 minutes. GST activity in each fraction was measured, and protein content was monitored at 280 nm. The fractions exhibiting high GST activity were collected and stored for subsequent analyses. For The kinetic properties of GST were examined under standard reaction conditions. The effect of enzyme concentration was determined by using purified GST at concentrations ranging from 5 to 40 mg/mL, with distilled water used to adjust the final volume. The effect of pH was assessed by performing the enzymatic reaction with fractions eluted from the Sephadex G-75 column at different pH values (4–8) while maintaining a constant buffer concentration; the reaction rate was plotted against pH to determine the optimum value. The effect of temperature was evaluated by conducting the reaction at different temperatures (10–45°C) in 0.1 M phosphate buffer (pH 6.25), and enzyme activity was recorded at each condition to establish the optimal temperature for GST activity (Nama et al., 2023: Kaky et al., 2025).

RESULT

Partial Purification of GST

Glutathione-S-Transferase (GST) was partially purified from the serum of leukemia patients using ammonium sulfate fractionation, dialysis, and Sephadex G-75 gel filtration chromatography (Table and Figure). The initial crude serum extract showed a protein concentration of 5.7 mg/mL with a specific activity of 0.1947 U/mg. After 75% ammonium sulfate precipitation, specific activity increased to 0.2805 U/mg with a recovery of 72.79%. Subsequent dialysis further enhanced the specific activity to 0.3932 U/mg with a yield of 69.07 %. The highest purification was achieved after gel filtration (fraction B), where the specific activity reached 1.888 U/mg, corresponding to a 9.698-fold purification and a final yield of 86.75%. These results confirm effective enrichment of GST from serum and removal of contaminating proteins.

Table 1: -Purification Step of Glutathione-S-Transferase in Serum of Leukemia

Purification Step		Volume mL	Protein Con. mg/mL	Total Protein mg	Enzyme Activity U/mL	Total Activity IU	Specific Activity U/mg	Fold of Purification	Yield 100%
Serum		10	5.7	57	1.11	11.1	0.1947	1	100%
Salt Precipitation Ammoniumsulfate75 %		8	3.6	28.8	1.01	8.08	0.2805	1.4409	72.79%
Dialysis		7.8	2.5	19.5	0.983	7.6674	0.3932	2.0195	69.07%
Gel Filtration Sephadex G-75	**A**	12	0.33	3.96	0.363	4.356	1.1	5.656	39.24%
	B	15	0.34	5.1	0.642	9.63	1.888	9.698	86.75%

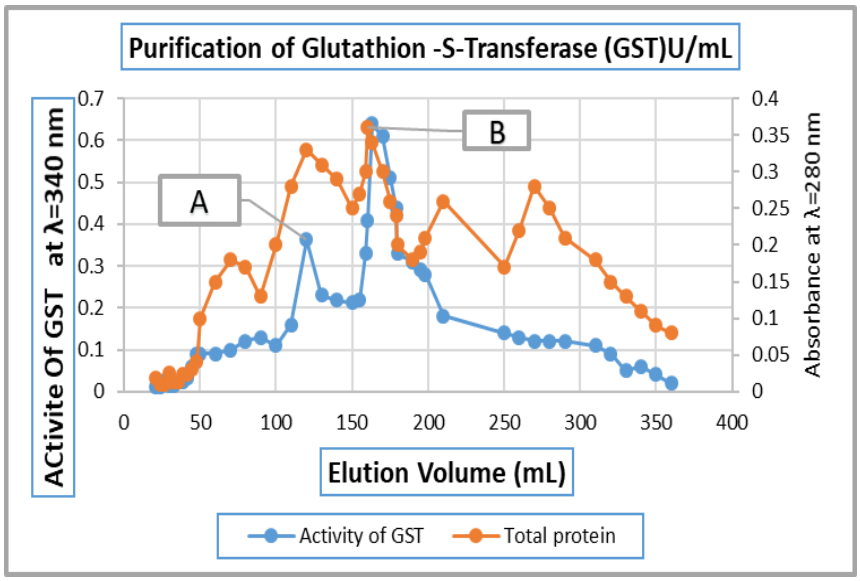

FIGURE 1. Elution Volumes of the Protein Fraction for The GST Enzyme in Blood Serum Obtained from The Gel Filtration Chromatography Column

Kinetic Properties of GST Enzyme

Enzyme activity was evaluated under different conditions to determine the optimum catalytic environment. GST displayed maximal activity at pH 6.25 (figure 2), buffer concentration of 0.12 M (figure 3), and temperature of 25 °C (figure 4). These results suggest that GST from leukemic serum maintains catalytic efficiency under slightly acidic and lower-than-physiological conditions, indicating possible conformational alterations associated with leukemic oxidative stress.

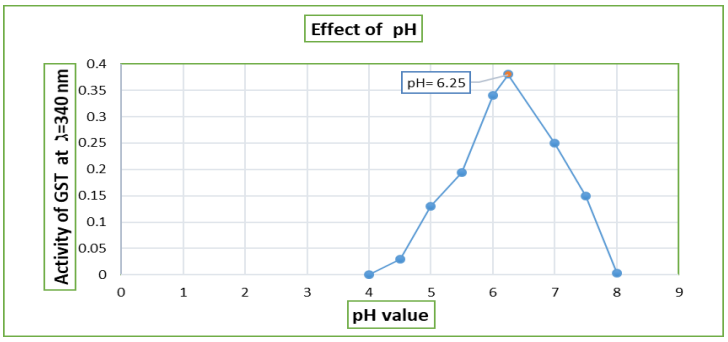

FIGURE 2. -Effect of pH on the GST Activity

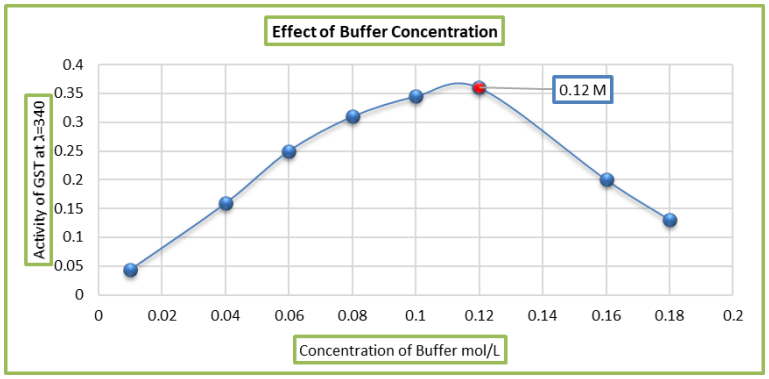

FIGURE 3. Effect of Buffer Concentration on GST Activity

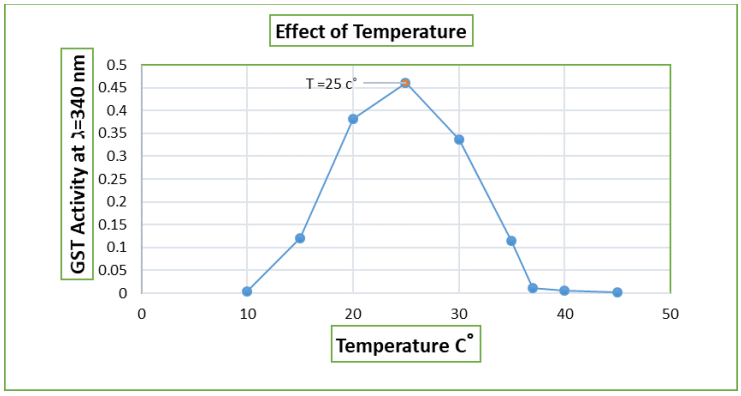

FIGURE 4. Effect of Temperature on the Activity of Glutathione -S-Transferase

Antioxidant Biomarkers in Patients Versus Controls

A significant reduction in antioxidant parameters was observed in leukemia patients compared with healthy controls. Mean catalase activity was (92.47 ± 12.93 mk/L) in patients versus (112.78 ± 15.51 mk/L) in controls ($p \leq 0.05$). Reduced glutathione levels decreased from (15.8 ± 2.26 µmol/L in controls to 10.1 ± 1.33 µmol/L) in patients ($p \leq 0.05$). Total antioxidant capacity (TAC) showed the most pronounced decline, from (352.8 ± 52.80 µmol/L in controls to 196.6 ± 30.81 µmol/L) in patients ($p \leq 0.05$), table 2.

Table 2: Concentration of (TAC, GSH, CAT) in the Serum of Patients Compare to the Control

Parameters	Mean± SD	
	Patient n =107	Control n =65
CAT Cat (mk/L)	92.47±12.93	112.78±15.51
GSH µmol/L	10.1±1.33	15.8±2.26
TAC µmol/L	196.6±30.81	352.8±52.80

Antioxidant Status by Disease Type

When stratified by clinical subtype, both acute and chronic leukemia patients showed lower antioxidant biomarkers than their respective controls. Acute leukemia patients exhibited the lowest values, with CAT activity (90.52 ± 16.19 mk/L) and TAC (189.4 ± 25.03 µmol/L) markedly reduced compared with controls (116.56 ± 16.92 and 377.9 ± 56.97 µmol/L, respectively). Chronic patients retained relatively higher antioxidant levels (CAT: 97.65 ± 16.02 mk/L, TAC: 225.7 ± 32.02 µmol/L), though still significantly below controls, table 3.

TABLE 3. Concentration of (TAC, GSH, CAT) in the Serum of Patients with Leukemia Compared to Control Group Depended on Type

Type	Chronic patients		Acute patients	
	Mean± SD		Mean± SD	
Parameters	Patient n =50	Control n=25	Patient n =57	Control n=40
CAT Ca(mK/L)	97.65±16.02	106.48±18.22	90.52±16.19	116.56±16.92
GSH µmol/L	9.79±1.63	13.9±1.56	10.22±1.68	16.88±2.36
TAC µmol/L	225.7±32.02	343.1±44.07	189.4±25.03	377.9±56.97

Antioxidant Status by Sex

Analysis by sex revealed differences in antioxidant depletion. Female patients had lower TAC (191.6 ± 33.81 µmol/L) compared to male patients (223.45 ± 32.22 µmol/L), though both groups were significantly reduced compared with their respective controls (413.5 ± 53.16 µmol/L in females and 337.1 ± 52.03 µmol/L in males). GSH was also reduced in both sexes but showed slightly higher preservation in males (10.13 ± 1.31 µmol/L) versus females (9.94 ± 1.35 µmol/L).

TABLE 5. Concentration of (TAC, GSH, MDA, CAT) in the serum of Patients with Leukemia Compared to Control Group Depended on Sex

Sex	Female		Male	
	Mean± SD		Mean± SD	
Parameters	Patient n =52	Control n=32	Patient n =55	Control n=33
CAT Cat(mK/L)	90.63±13.05	115.84±17.02	93.03 ± 12.89	108.06±13.28
GSH µmol/L	9.94±1.35	14.4±2.34	10.13±1.31	16.32±2.19
TAC µmol/L	191.6±33.81	413.5±53.16	223.45±32.22	337.1±52.03

Antioxidant Status by Age and Leukemia Subtype

Age- and subtype-specific analysis revealed distinctive patterns. Younger ALL patients (G1, ages 9–25) showed paradoxically higher GSH levels (10.71 ± 1.67 µmol/L) compared with their controls (8.27 ± 1.79 µmol/L), despite reduced CAT and TAC. In contrast, AML (G2) and CML (G4) patients demonstrated marked reductions across all biomarkers, particularly GSH (G2: 9.26 ± 1.60 µmol/L vs control: 13.7 ± 2.11 µmol/L) and TAC (G2: 186.6 ± 32.07 µmol/L vs control: 338.2 ± 43.42 µmol/L). CLL patients (G3) retained relatively higher antioxidant activity (CAT: 100.11 ± 18.24 mk/L, TAC: 253.3 ± 30.8 µmol/L) compared to AML and CML subtypes.

TABLE 5. Concentration of (TAC, GSH, CAT) in the Serum of Patients with Leukemia Compared to Control Group Depended on Age Group

Parameter	Age	Patient ALL=G1 n=30 AmL=G2 n=27 CLL=G3 n=25 CmL=G4 n=25	Control G1 n=20 G2 n=20 G3&G4 n=25
		Mean± SD	Mean± SD
CAT Cat(mK/L)	G1(9-25)	96.775±13.28	121.013±19.87
	G2(26-45)	86.64±12.51	115.86±20.45
	G3(46-65)	100.11±18.24	106.48±18.22
	G4(46-65)	93.03±17.99	106.48±18.22
GSH µmol/L	G1(9-25)	10.71±1.67	18.27±1.79
	G2(26-45)	9.26±1.599	13.7±2.11
	G3(46-65)	10.72±1.42	13.9±1.56
	G4(46-65)	8.75±1.301	13.9±1.56
TAC µmol/L	G1(9-25)	194.25±34.34	417.55±49.86
	G2(26-45)	186.6±32.07	338.2±43.42
	G3(46-65)	253.3±30.8	343.1±45.85
	G4(46-65)	222.1±31.007	343.1±45.85

DISCUSSION

In the present study, Glutathione-S-Transferase (GST) was successfully partially purified from the serum of leukemia patients using a classical multi-step purification approach. Employing ammonium sulfate precipitation together with dialysis and gel filtration chromatography resulted in a 9.698-fold purification and 86.75% yield, accompanied by a final specific activity of 1.888 U/mg. The present findings are in line with earlier reports describing GST derived from serum or tissue achieved 6–12-fold enrichment using similar chromatographic approaches (Nama et al., 2023; Al-Dabbagh et al., 2020). The progressive increase in specific activity following dialysis and Sephadex G-75 separation highlights the effectiveness of gel filtration in removing contaminating proteins while retaining enzymatic activity. The kinetic properties of GST in leukemic serum demonstrated maximum catalytic efficiency at pH 6.25, buffer concentration of 0.12 M, and temperature of 25 °C. While most GST isoforms typically exhibit optimal activity near physiological pH (7.0–7.5) and temperatures close to 37 °C (Hayes & Strange, 2020), the shift towards a slightly acidic pH and lower temperature in our study may reflect conformational modifications of GST under leukemic oxidative stress. Structural alterations of GST have been reported in malignancies due to post-translational modifications, mutations, and ROS-mediated oxidation, which can change substrate affinity and catalytic turnover (Reuter et al., 2019). Such adaptations may enable leukemic cells to sustain detoxification processes under hostile oxidative conditions. Analysis of antioxidant biomarkers (CAT, GSH, TAC) revealed a clear reduction in leukemia patients compared with controls. CAT activity was reduced by ~18%, GSH by ~36%, and TAC by ~44%. This pronounced depletion underscores the redox imbalance and oxidative stress burden in leukemia, corroborating previous studies where decreased antioxidant enzyme activity was associated with poor prognosis and chemo resistance (Khan et al., 2021; Nunes et al., 2022). Reduced catalase impairs hydrogen peroxide detoxification, while depletion of GSH diminishes both conjugation reactions mediated by GST and the overall redox-buffering capacity of cells. The significant reduction in TAC further emphasizes systemic oxidative exhaustion in leukemia patients. Subgroup analysis provided additional insights. Acute leukemia patients exhibited the most severe antioxidant depletion, particularly in TAC levels, compared with chronic patients. This may be explained by the higher metabolic and proliferative rate in acute leukemia, which elevates ROS generation and rapidly depletes antioxidant reserves (Nunes et al., 2022). Chronic leukemia patients retained relatively higher antioxidant levels, suggesting a slower disease progression with less acute oxidative demand. Sex- and age-based comparisons revealed biologically relevant differences. Female patients showed disproportionately lower TAC compared with males, which could be influenced by hormonal regulation of antioxidant systems and differences in metabolic profiles. Interestingly, younger ALL patients displayed preserved or even elevated GSH levels compared with controls, potentially reflecting a

compensatory upregulation of glutathione metabolism in the early stages of disease. In contrast, individuals in the elderly group presenting with AML" and CML demonstrated profound reductions across all antioxidants, supporting the hypothesis that disease subtype, age, and disease chronicity strongly influence redox status.

CONCLUSION

In summary, partial purification of GST from leukemia patient serum demonstrated effective enrichment and retention of enzymatic activity. GST exhibited maximum catalytic efficiency at slightly acidic pH and moderate temperature, reflecting adaptive responses to oxidative stress. Antioxidant biomarkers (CAT, GSH, TAC) were markedly reduced, particularly in acute leukemia, highlighting systemic oxidative imbalance. Age, sex, and leukemia subtype influenced antioxidant status, with younger ALL patients showing compensatory GSH preservation, while older AML and CML patients displayed profound depletion. These findings emphasize the dual role of GST as a detoxification enzyme and as a sensitive indicator of redox disturbances in leukemia.

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