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Mineral Pitch Attenuates Oxidative Stress-Induced Eryptosis in Human Erythrocytes via Antioxidants and Calcium-Modulatory Mechanism

Muhammad Najamul Hassan[1], Muhammad Rasheed[1], Namra Butt[1], Nida Anwar[2], Ahmad Shahab[1], Maryam Ashiq[1], Wazir Hamid Ali[3], Kashif Jilani[1]

[1]Department of Biochemistry, University of Agriculture, Faisalabad, Punjab, Pakistan.
[2]Department of Botany, University of Agriculture, Faisalabad, Punjab, Pakistan.
[3]Department of Biochemistry, Government College University Faisalabad, Punjab, Pakistan.

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Correspondence to: Kashif Jilani, Department of Biochemistry, University of Agriculture, Faisalabad, Punjab, Pakistan.
Email: kashif.jillani@uaf.edu.pk

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Muhammad Najamul Hassan and Muhammad Rasheed: Conceptualized and wrote the manuscript. All other authors contributed to the study and approved the final manuscript

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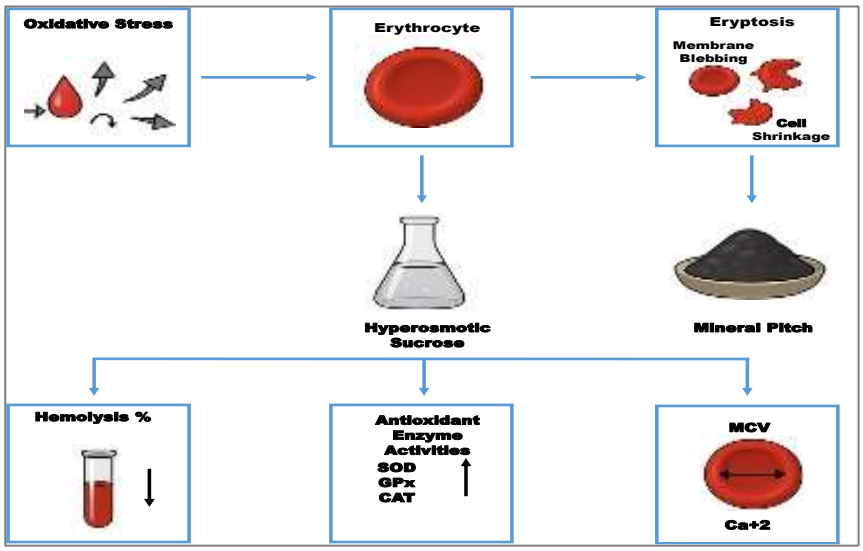
ABSTRACT

Background: Erythrocytes are highly susceptible to oxidative stress, which can induce eryptosis programmed cell death mechanism characterized by hemolysis, membrane blebbing, and cell shrinkage. Mineral Pitch (Asphaltum punjabium), a traditional medicinal substance, has demonstrated antioxidant potential, but its effect on erythrocyte integrity under oxidative stress has not been thoroughly investigated. **Objective:** This study aimed to evaluate the antioxidant and cytoprotective effects of Mineral Pitch on human erythrocytes exposed to oxidative stress induced by hyperosmotic sucrose. **Methods:** Human erythrocytes were treated with sucrose (350–550 mM) to induce oxidative stress and then exposed to Mineral Pitch at concentrations of 0.5, 1.0, and 1.5 g/10 mL. Hemolysis percentage, antioxidant enzyme activities (SOD, GPx, and CAT), mean corpuscular volume (MCV), and calcium channel involvement were analyzed using standard biochemical assays and ELISA. **Results:** Sucrose elevated hemolysis from 0.16% to 0.24%, while Mineral Pitch significantly reduced it to 0.16% at 1.5 g/10 mL. GPx activity, which decreased to 213.8 U/gHb under stress, was restored to 335 U/gHb. SOD activity improved from 877 U/gHb to 794 U/gHb, and CAT activity increased from 34.6 U/gHb to 44 U/gHb following treatment. MCV dropped to 68 fL with sucrose but was preserved at 85 fL with Mineral Pitch. Furthermore, the use of amlodipine confirmed a calcium-dependent mechanism in eryptosis, modulated by Mineral Pitch. **Conclusion:** Mineral Pitch demonstrates potent antioxidant and cytoprotective effects on erythrocytes under oxidative stress. It stabilizes cell membranes, restores antioxidant defenses, and regulates calcium-mediated eryptosis. These findings suggest its potential as a natural therapeutic agent against oxidative hematological damage.

Graphical Abstract



INTRODUCTION

Blood is a complex specialized fluid consisting of plasma (54%), erythrocytes (45%), leukocytes (0.7%), and platelets, constituting about 6–8% of total body mass [1]. Among these, erythrocytes (red blood cells, RBCs) are the most abundant and play a critical role in oxygen and carbon dioxide transport through hemoglobin, an iron-containing protein [2]. Unlike typical cells, erythrocytes lack nuclei and mitochondria and derive their energy solely via anaerobic glycolysis (Embden-Meyerhof pathway) [3].

Erythropoiesis, the continuous production of erythrocytes at a rate of approximately two million cells per second, is regulated by erythropoietin signaling in the bone marrow [4-6]. The characteristic biconcave, deformable structure of erythrocytes facilitates efficient gas exchange and passage through narrow capillaries [7]. This deformability is maintained by a lipid bilayer membrane supported by a cytoskeletal network, ensuring mechanical resilience and longevity in circulation, typically around 120 days [8, 9]. Senescent erythrocytes are cleared by macrophages, with iron recycled by transferrins to maintain homeostasis [10]. Erythropoiesis proceeds from hematopoietic stem cells through committed erythroid progenitors (CFU-Mix, BFU-E, CFU-E) to reticulocytes and mature erythrocytes, regulated by oxygen-sensing mechanisms and erythropoietin [11, 12]. During circulation, erythrocytes face various physiological stresses such as shear forces, osmotic fluctuations, and mechanical deformation [13]. Their survival under these conditions depends on maintaining membrane lipid asymmetry and regulating ion channels [14, 15].

Oxidative stress is a major factor contributing to erythrocyte damage and eryptosis (programmed RBC death), implicated in numerous pathologies including cancer, neurological diseases, atherosclerosis, and hypertension [16-18]. It arises from an imbalance between reactive oxygen species (ROS) generation and antioxidant defenses, resulting in oxidative damage to proteins, lipids, DNA, and RNA [19]. Erythrocytes are particularly vulnerable to ROS, such as superoxide anion and hydrogen peroxide, generated endogenously or from external sources. To mitigate oxidative injury, RBCs rely on enzymatic antioxidants (superoxide dismutase [SOD], catalase, glutathione peroxidase) and non-enzymatic antioxidants (glutathione, thiols, flavonoids, vitamins) [20].ROS encompasses free radicals and their reactive intermediates, with superoxide anion, primarily mitochondrial in origin, being the most prevalent [21]. It can interact with nitric oxide to form peroxynitrite, a potent oxidant capable of inducing cellular damage [22, 23]. Both enzymatic systems (e.g., NADPH oxidase, cytochrome P450) and non-enzymatic factors (e.g., radiation, pollution, metals, drugs) contribute to ROS and reactive nitrogen species (RNS) production [24, 25]. Oxidative stress leads to lipid peroxidation, generating cytotoxic byproducts like malondialdehyde and impairing protein function by structural modification [26, 27]. While physiological levels of ROS are important for signaling and immune defense, their excess causes cellular dysfunction and death [28, 29]. Antioxidants play a crucial role in neutralizing ROS, thus protecting cells from oxidative damage [30, 31].

Mineral Pitch, also known as *Asphaltum punjabium*, is a tar-like, sticky substance derived from high-altitude rocks in regions such as the Karakoram, Himalayas, Tibet, and Gilgit Baltistan, formed by the slow decomposition of plant material over centuries [32, 33]. Its complex composition includes plant-derived humic substances and microbial compounds from rock rhizospheres [34]. Traditionally valued in Ayurvedic and folk medicine as an adaptogen and rejuvenator, Mineral Pitch exhibits antioxidant, anti-inflammatory, anti-hyperglycemic, and regenerative properties [35, 36]. It has demonstrated therapeutic potential in managing osteoporosis, ulcers, diabetes, kidney stones, and cardiovascular disorders [37].Given the critical role of oxidative stress in erythrocyte damage and the promising pharmacological profile of Mineral Pitch, this study aims to investigate its effects on erythrocyte oxidation and eryptosis in vitro. Furthermore, it evaluates the antioxidant potential of Mineral Pitch and explores calcium's role in oxidative stress-induced programmed erythrocyte death.

MATERIALS AND METHODS

Preparation of Mineral Pitch Solution

Mineral pitch solutions with different concentrations was prepared by dissolving 0.5g, 1g and 1.5g in 10ml of distilled water. Mineral Pitch is completely dissolved in distilled water.

Preparation of Sample

Samples were prepared from the erythrocytes obtained from blood. In these samples, 2 control and 3 treatments were used in which 1ml ringer, 4µl blood were added. In Treatment 1, 2 and 3, 1 µL of various concentrations of mineral pitch 0.5g/10ml, 1g/10ml and 1.5g/10 ml were added.

Hemolysis Measurement

Both sorts of samples were incubated at 37^{0}C for 48 hours, after which they were micro-centrifuged at 3000rpm for 3 minutes. Supernatant, possessing hemoglobin, were collected and hemolysis percentage was determined from it, in a spectrophotometer at 560 nm [38].

Measurement of Oxidative Stress

Erythrocytes naturally possess various antioxidants to counter ROS formed in them. Various anti-oxidant enzymes, carrier proteins, trapping molecules and a pharmacological defence system are present in the body. The defense molecules include Vitamins (A and D), and GSH, while enzymes include superoxide dismutase (SOD), glutathione peroxidase (GP_x) and Catalase (CAT). In addition, various phytochemicals possess anti-oxidant activity and thus can be used to reduce oxidative stress in cells [39].

Superoxide Dismutase

SOD activity was measured to access anti-oxidant potential of cell. Riboflavin and methionine served as source of superoxide production, which then either reduce NBT (which serve as source of chromophore), or get oxidized by SOD. The Eliza plate reader were used for

assessment of SOD activity at 570nm, in both kinds of sample [40].The lesser extent of NBT's photo-reduction corresponds with activity of SOD [27]

Reaction Mixture for SOD

The reaction mixture components of SOD were prepared as follow: 0.035g triton X-100 were dissolved in 15mL distilled water, 0.03g NBT were mixed in 30mL distilled water, 0.44g methionine were dissolved in 30mL distilled water, and 0.07g riboflavin in 30mL distilled water. All these solutions were mixed together in concentrations 800 µL Distilled water, 100µL NBT, 200µL Methionine, 200µL Triton-X and dissolve in 0.2M phosphate buffer.

Assay Procedure for SOD

Both sorts of samples were mixed with reaction mixtures in 96 well plate of Elisa, sample were poured in these wells under UV lamps, then riboflavin were added in these wells. SOD activity were assayed at 570nm in Elisa plate reader [41].

Reaction Mixture of Glutathione Peroxidase (POD)

20mM guaiacol, 40mM H_2O_2 and 0.2M buffer were mixed to get reaction mixture. Than absorbance were taken at 490nm in Elisa plate reader.

Assay Procedure for POD

To perform the POD assay, 50 µL of each sample was combined with 50 µL of the POD reaction mixture in separate wells of a 96-well plate. Once all the samples were prepared and the enzyme solution was added, the plates were placed in an ELISA plate reader to measure absorbance at 490 nm. This procedure was consistently followed for samples treated with varying concentrations of sucrose as well as those exposed to different concentrations of Mineral Pitch [42].

Reaction Mixture for Catalase

H_2O_2 were mixed with 0.2M phosphate buffer and introduced in sample. The absorbance were measured at 240nm in Elisa plate reader.

Assay Procedure for CAT

For the CAT assay, 50 µL of each sample was mixed with 50 µL of the CAT reaction mixture in individual wells of a 96-well plate. After adding the enzyme solution to all samples, the plates were placed in an ELISA plate reader to record the absorbance at 240 nm. This method was uniformly applied to samples treated with varying concentrations of sucrose as well as those treated with different concentrations of Mineral Pitch [43].

MCV Measurement

Shrinkage of erythrocytes and membrane blebbing are characteristic features of eryptosis. Mean corpuscular volume were calculated via automated hematology analyzer [42].

Calcium Role Confirmation

Amlodipine is a chelator of calcium channel. Erythrocytes showed eryptosis when exposed with toxins in presence of calcium. Erythrocytes were than treated with toxins in presence of amlodipine, a calcium channel blocker. The calcium channel activity were confirmed by antioxidant enzyme assay [27].

Statistical Analysis

Statistical analysis was carried out using analysis of variance (ANOVA), followed by Tukey's post-hoc test. ANOVA was used to assess the overall significance between the control and treatment groups, while Tukey's test helped identify specific differences among them. The experimental design included a negative control, a positive control, and three treatment groups. Each experiment was repeated more than 10 times to ensure accuracy and reliability.[44].

Results and Discussion

Hemolysis Assessment

Eryptosis is a programmed death mechanism in erythrocytes, often triggered by oxidative stress, and is characterized by membrane blebbing, shrinkage, and ultimately hemolysis. The increase in hemolysis observed with escalating sucrose concentrations confirms its pro-oxidant role (**Table 3.1**;**Figure 3.1)**. Sucrose disrupts red cell membranes by generating reactive oxygen species (ROS), leading to leakage of hemoglobin and increased hemolytic index.

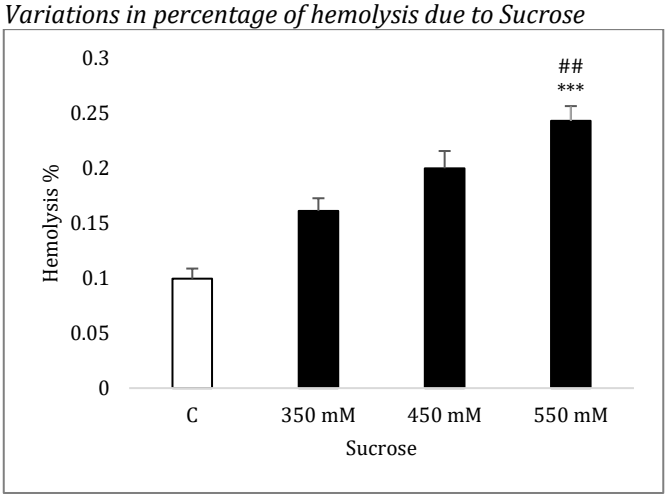
Table 3.1

Variations in percentage of hemolysis due to Sucrose

Blood samples	Control	350 mM	450 mM	550 Mm
1	0.087	0.115	0.342	0.381
2	0.114	0.132	0.287	0.195
3	0.091	0.152	0.127	0.224
4	0.118	0.212	0.258	0.287
5	0.107	0.115	0.211	0.216
Average	0.09960	0.16126	0.20006	0.24326
SD	0.03561	0.04471	0.06091	0.05191
SEM	0.009194	0.01154	0.01573	0.01340

Figure 3.1

Variations in percentage of hemolysis due to Sucrose



The data are presented as arithmetic means ± SEM (n = 15). A graph was plotted with hemolysis percentage on the Y-axis and varying sucrose concentrations on the X-axis. The control group, which did not receive sucrose, is represented by a white bar, while the three treatment groups with different sucrose concentrations are shown as black bars. The control sample contained 1000 µL of Ringer's solution and 4 µL of blood. Each treatment group also included blood and Ringer's solution, along with different sucrose concentrations: T1 (350 mM), T2 (450 mM), and T3 (550 mM). All samples were incubated for 48 hours. Error bars on the graph represent the SEM.

Statistically significant differences between treated and untreated samples are indicated as ** ($p < 0.01$), ## ($p < 0.01$), and *** ($p < 0.001$). The results demonstrated a clear trend: hemolysis percentage increased with rising sucrose concentrations.

Treatment with Mineral Pitch significantly reduced hemolysis dose-dependently (**Table 3.2**; **Figure 3.2**), suggesting that its bioactive constituents may stabilize the erythrocyte membrane or counteract ROS production.

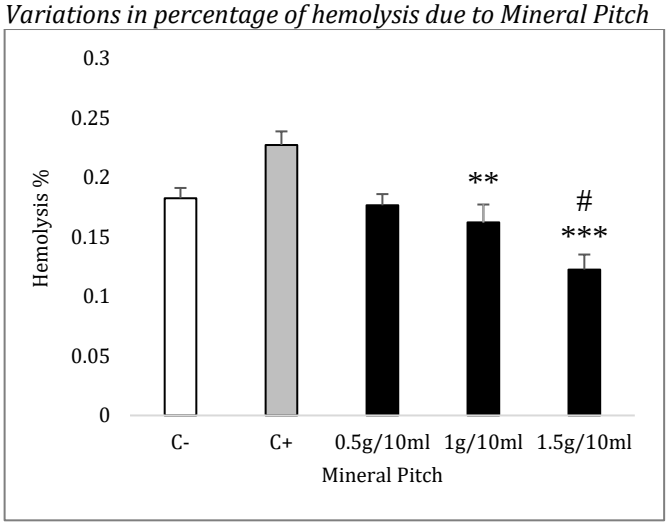
Table 3.2

Variations in percentage of hemolysis due to Mineral Pitch

Blood samples	C-	C+	0.5g/10ml	1g/10ml	1.5g/10ml
1	0.087	0.151	0.115	0.342	0.381
2	0.114	0.122	0.132	0.287	0.195
3	0.091	0.162	0.152	0.127	0.224
4	0.118	0.154	0.212	0.258	0.287
5	0.107	0.165	0.115	0.211	0.216
Average	0.18246	0.26831	0.22720	0.176533	0.162133
SD	0.033554	0.018829	0.044372	0.036571	0.058377
SEM	0.008663	0.005435	0.011457	0.009442	0.015073

Figure 3.2

Variations in percentage of hemolysis due to Mineral Pitch



Arithmetic means ±SEM (n = 15) were estimated. Erythrocytes were treated with Mineral Pitch and incubated for about 48 hours in Ringer solution in the absence of Mineral Pitch (white bar), Sucrose as a positive control (grey bar) or in the presence of Mineral Pitch (black bar) with different concentrations. Standard error mean (SEM) is presented on Y-axis bars. The efficient variations in treated and non-treated cells showed when * ($p<0.05$) and *** ($p<0.001$) (ANOVA) while ### ($p<0.001$) shows a significant difference in both treatments.

This result is consistent with earlier findings that natural polyphenols and fulvic acids in Mineral Pitch exhibit antioxidative and cytoprotective activities [38].

Oxidative Stress Parameters

Oxidative stress is a central mechanism in eryptosis, impairing cellular redox balance and compromising antioxidant defense mechanisms.

Glutathione Peroxidase (GPx)

Glutathione peroxidase is vital in neutralizing hydrogen peroxide and lipid hydroperoxides in cells. The decline in GPx activity in sucrose-treated cells (**Table 3.3**; **Figure 3.3**) indicates oxidative overload. Conversely, Mineral Pitch enhanced GPx activity (**Table 3.4**;**Figure Figure 3.4**), especially at higher concentrations, indicating its efficacy in restoring redox balance. This effect may stem from the synergistic action of its mineral and organic constituents, which facilitate the regeneration of reduced glutathione or directly scavenge ROS.

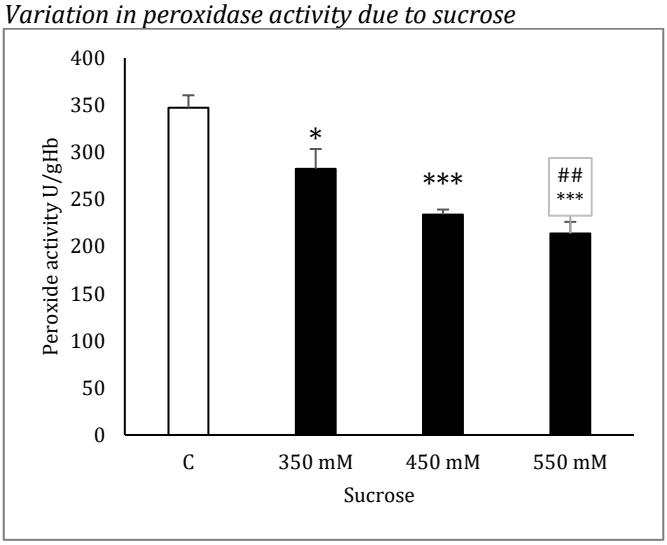
Table 3.3

Variation in peroxidase activity due to sucrose

Blood samples	C	350 mM	450 mM	550 Mm
1	400	229	217	245
2	289	224	223	218
3	293	238	260	228
4	353	246	222	244
5	360	376	226	253
mean	347.25	282.417	233.917	213.833
SD	45.7883	72.7792	18.3127	42.4881
SEM	13.2179	21.0095	5.28643	12.2653

Figure 3.3

Variation in peroxidase activity due to sucrose



The results are expressed as arithmetic means ± SEM (n = 12). A graph was plotted with peroxidase activity on the Y-axis and different sucrose concentrations on the X-axis. The negative control (without sucrose) is represented by a white bar, while the positive control (with sucrose) is shown as a grey bar. The three treatment groups are depicted as black bars. Error bars indicate the standard error of the mean (SEM). Statistically significant differences between groups are marked as * ($p < 0.05$) and *** ($p < 0.001$), indicating a notable difference between treated and untreated cells based on ANOVA. Additionally, ## ($p < 0.01$) denotes a significant variation between samples. The results revealed that peroxidase activity decreased as sucrose concentration increased.

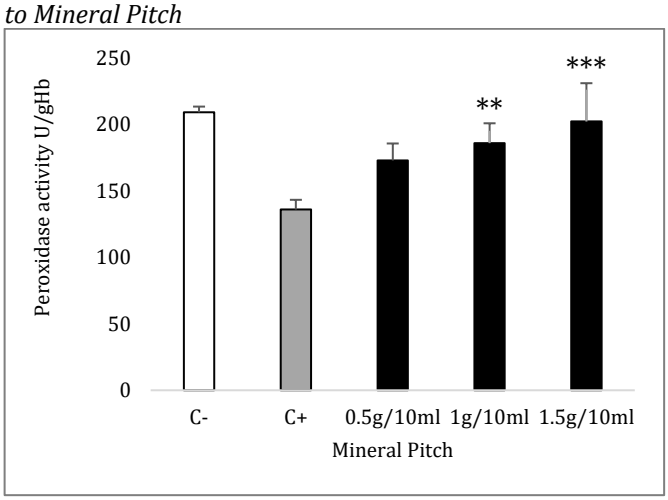
Table 3.4

Variations in glutathione peroxidase activities (U/gHb) due to Mineral Pitch

Blood samples	C-	C+	0.5g/10ml	1g/10ml	1.5g/10ml
1	199	121	225	246	100
2	213	104	178	108	309
3	221	136	110	162	330
4	236	119	220	262	335
5	194	110	212	131	335
SD	14.75943	25.15514	44.19872	51.61452	99.60923
SEM	4.260682	7.261663	12.75907	14.89983	28.75471

Figure 3.4

Variation in Glutathione peroxidase activities (U/gHb) due to Mineral Pitch



The data are presented as arithmetic means ± SEM (n = 15). Erythrocytes were treated with Mineral Pitch and incubated for 48 hours. After the incubation period, enzymatic activities were measured in both treated and untreated cells. The Y-axis displays the standard error of the mean (SEM). Statistically significant differences between groups are indicated as ** ($p < 0.01$) and *** ($p < 0.001$), reflecting notable differences between treated and untreated cells based on ANOVA. Additionally, ## ($p < 0.05$) denotes significant variation among treatment groups. As shown along the X-axis, exposure of isolated human erythrocytes to Mineral Pitch under specific conditions resulted in a decrease in GPx activity.

The analogous results were proposed about enzyme activity in literature [45].

Superoxide Dismutase (SOD)

SOD catalyzes the dismutation of superoxide radicals into oxygen and hydrogen peroxide. A reduction in SOD activity under sucrose stress (**Table 3.5**; **Figure 3.5**) demonstrates elevated oxidative damage. Treatment with Mineral Pitch partially restored SOD levels (**Table 3.6**; **Figure 3.6**), suggesting it supports the antioxidative defense system. The recovery of SOD activity reinforces the hypothesis that Mineral Pitch modulates intracellular oxidative signaling, thereby inhibiting eryptosis pathways.

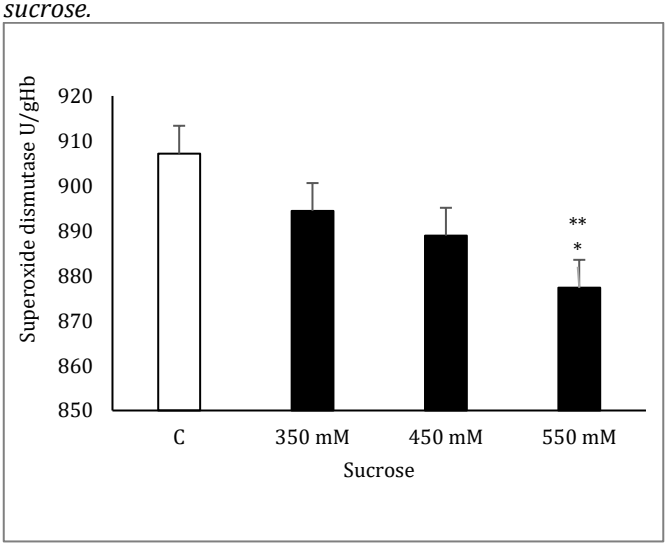
Table 3.5

Variations in superoxide dismutase activities due to sucrose

Blood samples	Control	350 mM	450 mM	550 Mm
1	895	880	890	887
2	896	870	922	879
3	869	874	860	869
4	950	914	880	902
5	931	920	875	883
Average	907.1666667	894.4166667	888.9166667	877.3333333
SD	28.51421761	26.37649895	23.32559395	27.63177793
SEM	8.231345607	7.614239385	6.733518974	7.976607213

Figure 3.5

Variation in superoxide dismutase activities (U/gHb) due to sucrose.



The results are presented as arithmetic means ± SEM (n = 12). A graph was plotted with SOD (superoxide dismutase) activity on the Y-axis and sucrose concentration on the X-axis. Erythrocytes were incubated for 48 hours in Ringer's solution with blood, either without sucrose (white bar) or with sucrose at concentrations of 350 mM, 450 mM, and 550 mM (black bars). After incubation, enzymatic activity was measured in both treated and untreated cells. Error bars on the Y-axis represent the standard error of the mean (SEM). A statistically significant difference between treated and untreated samples is indicated by ($p < 0.05$), as determined by ANOVA. The results indicate that superoxide dismutase activity decreases as sucrose concentration increases..

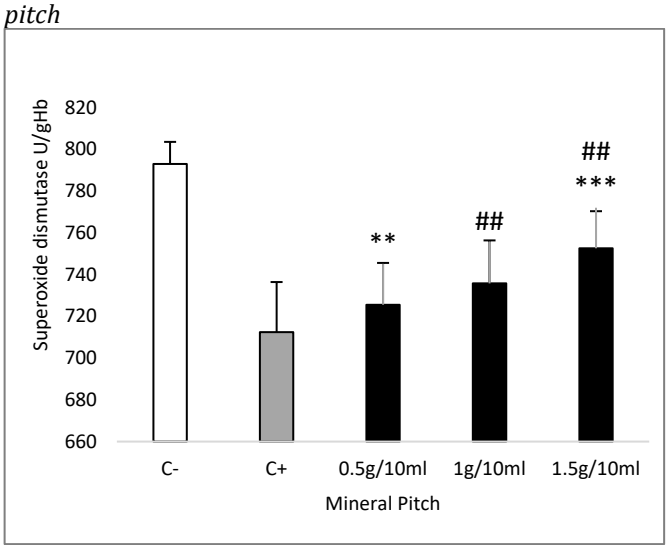
This experiment explored how varying concentrations of sucrose affect the activity of the antioxidant enzyme superoxide dismutase (SOD) in erythrocytes. The findings revealed a clear decline in SOD activity as sucrose levels increased. This suggests that higher sucrose concentrations may impair the antioxidant defense system of erythrocytes, reducing their capacity to counteract harmful reactive oxygen species (ROS). These results highlight a potential link between elevated sucrose exposure and weakened cellular resistance to oxidative stress. Further studies are needed to uncover the underlying mechanisms and assess the broader implications for cell health and oxidative stress-related disorders [45].

Table 3.6

Variations in Superoxide dismutase activities (U/gHb) due to Mineral pitch

Blood Samples	C-	C+	0.5g/10ml	1g/10ml	1.5g/10ml
1	726	811	686	642	794
2	786	558	691	640	799
3	742	677	686	675	696
4	772	634	621	659	683
5	842	755	637	764	764
SD	36.63512	83.08315	69.33707	71.35623	61.52155
SEM	10.57565	23.98404	20.01589	20.59877	17.75974

Figure 3.6
Variation in superoxide dismutase (U/gHb) due to Mineral pitch



The data are presented as arithmetic means ± SEM (n = 12). Erythrocytes were treated with Mineral Pitch and incubated for 48 hours. After the incubation period, enzymatic activities were assessed in both treated and untreated cells. Error bars on the Y-axis represent the standard error of the mean (SEM). Statistically significant differences between groups are indicated as ** ($p < 0.01$) and *** ($p < 0.001$), showing notable differences based on ANOVA, while ## ($p < 0.05$) represents significant variation among treatment groups. As shown along the X-axis, exposure of isolated human erythrocytes to Mineral Pitch under specific conditions led to a decrease in superoxide dismutase (SOD) activity.

Same results were also examined in which significant reduction in endogenous oxidative enzyme SOD [46].

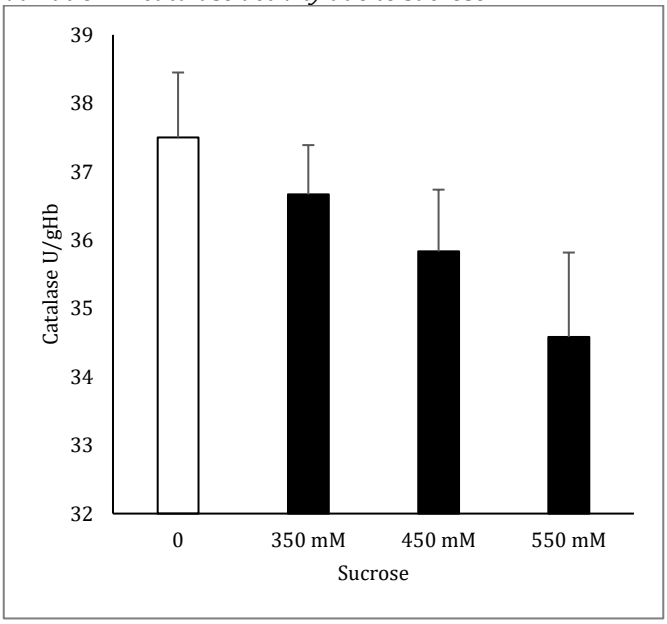
Catalase (CAT)

Catalase converts hydrogen peroxide into water and oxygen, serving as a secondary defense to SOD. As seen in previous parameters, catalase activity decreased significantly with sucrose exposure (**Table 3.7**;**Figure 3.7**), aligning with oxidative damage. Mineral Pitch treatments counteracted this suppression, particularly at 1.5 g/mL (**Table 3.8**;
Figure 3.8), indicating enhanced ROS detoxification. These effects support the antioxidant synergy between GPx, SOD, and catalase, orchestrated under the influence of Mineral Pitch.

Table 3.7
Variation in catalase activity due to sucrose

Blood samples	0	350 mM	450 mM	550 Mm
1	39	38	37	33
2	39	37	33	37
3	39	38	38	35
4	29	37	37	33
5	40	38	40	37
Mean	37.5	36.66667	35.83333	34.58333
SD	3.2891005	2.498484	3.128559	4.273775
SEM	0.9494815	0.72125	0.903137	1.233733

Figure 3.7
Variation in catalase activity due to sucrose



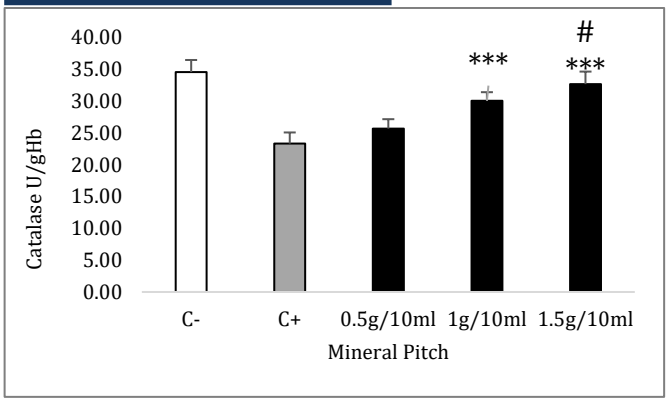
The results are expressed as arithmetic means ± SEM (n = 12). Erythrocytes were treated with sucrose and incubated for 48 hours in Ringer's solution with blood—either without sucrose (white bar) or with sucrose at concentrations of 350 mM, 450 mM, and 550 mM (black bars). After incubation, enzymatic activities were measured in both treated and untreated cells. The Y-axis displays the standard error of the mean (SEM). The results demonstrated that the activity of the antioxidant enzyme catalase decreased progressively with increasing sucrose concentrations.

The presented results demonstrate a significant impact of sucrose concentration on the activity of the antioxidative enzyme catalase in erythrocytes. The experiment revealed a clear trend of decreasing catalase activity with increasing sucrose concentration. The observed reduction in catalase activity could have important implications for cellular health and oxidative damage protection. These findings may contribute to our understanding of how sucrose intake or exposure could potentially impact the overall antioxidant capacity of erythrocytes and, consequently, the body's ability to mitigate oxidative stress.

Table 3.8
Variations in Catalase activities (U/gHb) due to Mineral pitch

Blood samples	C-	C+	0.5g/10ml	1g/10ml	1.5g/10ml
1	28	18	28	33	24
2	34	16	32	31	33
3	36	25	18	23	35
4	40	15	20	28	38
5	37	24	33	29	44
SD	5.80155	5.159959	4.708741	6.795822	7.675286
SEM	1.900791	1.744215	1.475014	1.350153	1.965687

Figure 3.8
Variation in Catalase activities (U/gHb) due to Mineral pitch



Data are presented as arithmetic means ± SEM (n = 12). Erythrocytes were treated with Mineral Pitch and incubated for 48 hours. The error bars on the Y-axis represent the standard error of the mean (SEM). Statistical analysis showed a highly significant difference between treated and untreated cells, indicated by *** ($p < 0.001$) based on ANOVA. As shown along the X-axis, exposure of isolated human erythrocytes to Mineral Pitch under specific conditions led to a decrease in catalase (CAT) activity.

The same results were also examined in which a significant reduction in endogenous oxidative enzyme CAT [47]

Mean Cell Volume (MCV) Analysis

Eryptosis is often accompanied by cell shrinkage due to ion loss, mainly K+ and Cl^-, followed by osmotic water loss. The decrease in MCV upon sucrose exposure (**Table 3.9**; **Figure 3.9)** is a hallmark of eryptotic cells. Mineral Pitch treatment preserved erythrocyte volume, highlighting its role in membrane and ionic homeostasis. This cytoprotective mechanism may involve the inhibition of cationic channels or the stabilization of membrane fluidity.

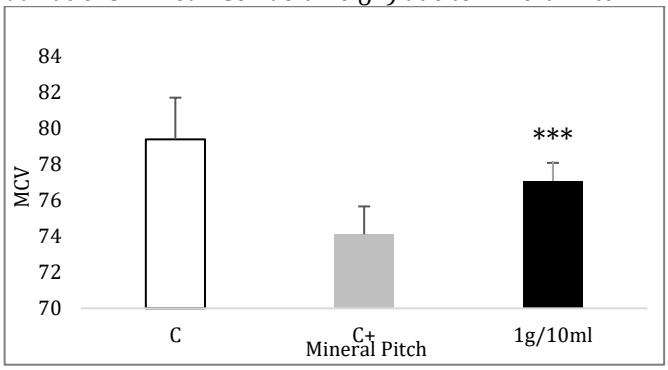
Table 3.9

Variations in mean cell volume due to Mineral pitch

Blood samples	C	C+	1 g/10ml
1	95.1	83.5	77
2	77.5	75.2	88
3	83.3	79.7	74
4	85	68.1	73
5	79.2	69.4	85
SD	8.965	6.010998	7.314044
SEM	2.315	1.552033	1.00765

Figure 3.9

Variations in Mean Cell Volume (fL) due to Mineral Pitch



Arithmetic means ±SEM (n = 15) were estimated. Erythrocytes treatment with Mineral Pitch and incubated for 48 hours after the sample centrifugation at 500 rpm for 3 minutes then supernatant calculated by spectrophotometer Y-axis bar show standard error means (SEM) *** ($p<0.001$) and ** ($p<0.01$) mention that, there is remarkable difference in treated and non-treated cells (ANOVA). As mentioned, along the X-axis with mineral pitch exposed to isolated human erythrocytes under specific conditions showed a decrease in mean cell volume.

Calcium's Role in Eryptosis Induction

Calcium influx is a key signal in eryptosis. The use of amlodipine, a calcium channel blocker, revealed that sucrose-induced eryptosis is calcium-dependent. Mineral Pitch increased calcium entry into erythrocytes (**Table 3.10**; **Figure 3.10**), supporting literature that attributes eryptosis induction to calcium-mediated pathways. However, in the presence of amlodipine, the effect was attenuated, reinforcing the hypothesis that Mineral Pitch interacts with calcium signaling cascades in a complex manner. Its constituents may modulate calcium channel activity either directly or through ROS-mediated secondary pathways.

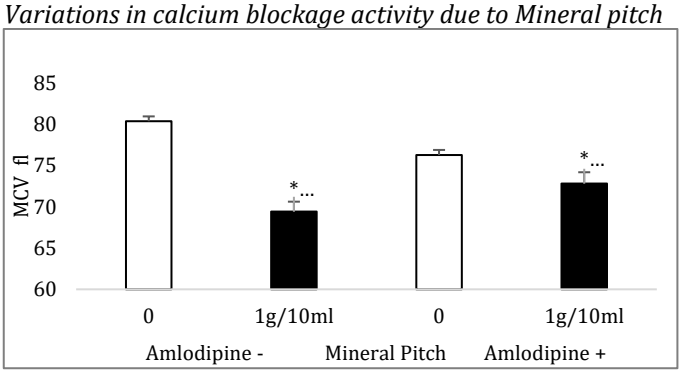
Table 3.10

Variations in calcium blockage activity due to Mineral Pitch

Blood samples	AMLODIPINE-		AMLODIPINE+	
	0	1g/10ml	0	1g/10ml
1	81.1	77.6	76.6	78.3
2	79.9	67.3	73.4	66.4
3	80.8	73.3	77.9	75.7
4	78.4	71.2	77.6	75
5	82.9	72.3	79.2	79.7
Average	80.32	69.4	76.23333333	72.78666667
SD	2.267534848	4.634497969	2.383774518	5.296611675
SEM	0.58547498	1.19662223	0.615487934	1.367579254

Figure 3.10

Variations in calcium blockage activity due to Mineral pitch



Arithmetic means ±SEM (n = 15) were estimated. Erythrocytes were treated with Mineral Pitch and incubated for 48 hours with Ringer solution in the absence of Mineral Pitch (white bar) or in the presence of Mineral Pitch (black bar). Standard error mean (SEM) is presented on Y-axis bars. The efficient variations in treated cells in the presence of amlodipine and amlodipine-insufficient cells showed when *** ($p<0.001$) (Tukey's test).

Human erythrocytes were treated with mineral pitch in the presence or absence of amlodipine with 1ml ringer solution for 48 hours at 37^0C. Mean cell volume reduce was determined. Literature had approved that mineral pitch act as stimulator to increase the calcium entry. Same results were also observed in which significant alteration in calcium blockage activity [48].

DISCUSSION

This study provides strong evidence that Mineral Pitch (Asphaltum punjabium), a traditional natural compound, can significantly protect erythrocytes from oxidative stress-induced damage. The exposure to hyperosmotic sucrose led to typical signs of eryptosis, including elevated hemolysis, reduced antioxidant activity, cell shrinkage, and calcium influx. However, treatment with Mineral Pitch showed a remarkable reversal of these effects, suggesting it plays a protective role by supporting redox balance and stabilizing cell membranes. Oxidative stress occurs when the production of reactive oxygen species (ROS) overwhelms the cell's antioxidant defenses, leading to structural and functional damage. In our study, erythrocytes treated with sucrose exhibited high levels of hemolysis, indicating membrane instability under oxidative pressure. Interestingly, Mineral Pitch significantly reduced this hemolysis in a dose-dependent manner. These findings may be attributed to the presence of active constituents like fulvic acids, polyphenols, and minerals, which are known to neutralize ROS and support membrane integrity. This observation is consistent with other studies on natural adaptogens that display antioxidant and membrane-stabilizing properties [30]

These results also draw a parallel with findings from [49], who demonstrated that the natural compound fluoxetine effectively protected erythrocytes from eryptosis by reducing oxidative stress and calcium influx. Although Mineral Pitch and piperlongumine differ in composition, both share the ability to counteract key mechanisms of eryptosis—namely, membrane destabilization, ROS accumulation, and calcium entry [49]. Our study supports this comparison by showing that Mineral Pitch, too, can interrupt these destructive processes in erythrocytes.

Additionally, our results showed that the antioxidant enzymes—glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT)—were significantly reduced under stress, but were restored close to normal levels following Mineral Pitch treatment. This suggests that Mineral Pitch not only scavenges free radicals but may also enhance the activity of the body's own antioxidant defenses. These enzymatic recoveries were accompanied by the preservation of mean corpuscular volume (MCV), indicating that Mineral Pitch helps prevent the characteristic cell shrinkage seen in eryptosis.

Another important aspect of this study is the role of calcium in mediating erythrocyte death. We observed that the influx of calcium—an established trigger for eryptosis—was significantly modulated by Mineral Pitch. When amlodipine, a calcium channel blocker, was introduced, the calcium influx was further suppressed. This indicates that Mineral Pitch may interfere with calcium entry directly or through ROS-related signaling. This finding is in line with previous research highlighting the close relationship between oxidative stress and calcium-mediated cell damage [17].

Together, these results suggest that Mineral Pitch is a promising natural agent for protecting erythrocytes from oxidative damage. Its multi-targeted actions—reducing ROS, restoring antioxidant enzyme activity, maintaining cell volume, and modulating calcium influx—point toward its potential therapeutic value in oxidative stress–related blood disorders. While our in vitro data are encouraging, future studies should focus on exploring the underlying molecular mechanisms, testing its efficacy in animal models, and assessing long-term safety and pharmacokinetics.

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