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Determination of the Role of Chrysin in Erythrocytes Survival

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Ismat Fatima and Masooma Haider conceptualized and wrote the manuscript. All other authors contributed to the study and approved the final manuscript

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ABSTRACT

Background: Oxidative and osmotic stress cause eryptosis, a type of programmed erythrocyte death known for its phosphatidylserine exposure and cell shrinkage. Sucrose-induced eryptosis is a good model to assess antioxidant treatments because of its oxidative damage. A naturally occurring flavonoid called chrysin has demonstrated encouraging anti-inflammatory and antioxidant capabilities. **Objectives:** The study of this work aimed to investigate how chrysin protects erythrocytes against hyperosmotic stress and eryptosis brought on by sucrose. The study specifically aimed to evaluate the levels of enzymes, hemolysis inhibition, cell size, and antioxidative activity in erythrocytes under stress. **Methods:** Human erythrocytes were subjected to varying chrysin and sugar concentrations for 48 hours. Mean cell volume, hemolysis %, hemolysis percentage, oxidative stress indicators (such as catalase and superoxide dismutase activity), and scanning electron microscopy for morphological assessment were all part of the experimental design. ANOVA was used to statistically examine the data. **Results:** The findings demonstrated that chrysin enhanced antioxidant enzyme activity, maintained cell volume, lowered hemolysis percentage, that result dramatically decreased eryptosis. When compared to stressed cells, SEM demonstrated that erythrocytes treated with chrysin retained their membrane integrity. The findings showed chrysin as an inhibitor for eryptosis. **Conclusion:** Chrysin's antioxidative properties protect erythrocytes by reducing eryptosis brought on by glucose-induced eryptosis. These findings reveal the protective mechanism of chrysin and suggest as a natural the therapeutic agent to reduce erythrocytes damage by oxidative stress and hyperosmotic shock.

INTRODUCTION

Red blood cells, or erythrocytes, are essential for delivering carbon dioxide and oxygen throughout the body [1]. With roughly 20 to 30 trillion in circulation, they make up one-fourth of all body cells and are the most prevalent type of cell in the human body [2]. Erythrocytes remain metabolically active and serve as important markers of physiological health, even in the absence of nuclei and mitochondria [3]. Erythrocyte counts in women range from 4 to 5 million per microliter of blood, whereas in men they range from 5 to 6 million [4]. The erythrocyte membrane is made up of three layers: the external glycocalyx, which is rich in carbohydrates; the lipid bilayer, which includes numerous trans membrane proteins; and membrane skeleton, which is located on the inner surface of the lipid bilayer. In human erythrocytes, half of the cell membrane mass is made up of proteins. The control of erythrocyte surface deformability, flexibility, adherence to other cells, and immunological recognition depends heavily on the erythrocyte membrane [5]

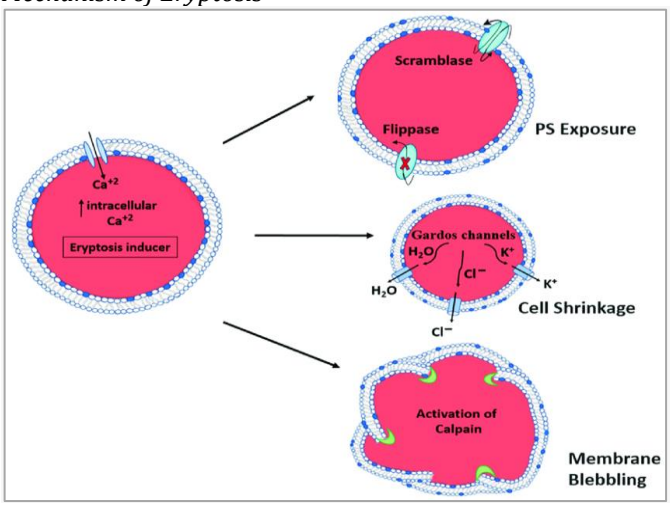
Erythropoiesis is the production of RBCs in the bone marrow. The kidney hormone erythropoietin (EPO) stimulates erythropoiesis [6]. Erythrocytes move through the bloodstream for 100–120 days earlier than macrophages eat them and replace the parts that make them up. [7]. Most of the time, each cycle takes 20 seconds. About 25% of the cells are red blood cells in the human body [8]. However, erythrocytes are vulnerable to membrane damage as a result of ongoing exposure to oxidative and osmotic stressors, which may cause hemoglobin leakage and even kidney toxicity [9].

The immune system and tissue homeostasis depend on apoptosis, a type of programmed cell death. It is characterized by shrinkage of cell, condensation of chromatin (pyknosis), and membrane blebbing, which are frequently brought on by immunological reactions or DNA

damage [10]. This energy-dependent mechanism, which is essential for growth, aging, and disease regulation, occurs through either internal (mitochondrial) or extrinsic (death receptor) routes [11]. Phosphatidylserine (PS) externalization, membrane shrinking, and blebbing are the hallmarks of eryptosis, a specific type of apoptosis in erythrocytes. The main causes of it include hyperosmotic shock, elevated intracellular calcium levels, oxidative stress, and energy depletion. Through scramblase activation and flippase inactivation, these stimuli open non-selective Ca^{2+} channels, exposing PS [12]. Cell shrinkage results from the activation of Gardos channels by elevated intracellular Ca^{2+}, which also causes K^+ and water loss. RBCs are further sensitized to Ca^{2+} by ceramide and platelet-activating factor (PAF), which intensifies eryptosis [13]. Additionally, eryptotic signaling is facilitated by a number of kinases and receptors, including CD47 and CD95/Fas. Erythropoietin (EPO), nitric oxide (NO), adenosine, and certain kinases are protective agents against eryptosis. NO inhibits Ca^{2+}-mediated pathways, whereas EPO prevents Ca^{2+} entry and oxidative stress-induced eryptosis. But through oxidative stress, too much NO donors can potentially cause eryptosis [14].

Figure 1.1

Mechanism of Eryptosis



When reactive oxygen and nitrogen species (ROS/RNS) overwhelm the antioxidant defense mechanism, oxidative stress results [15]. Chronic illnesses like diabetes, cancer, neurodegeneration, and cardiovascular disorders are all significantly impacted by this imbalance. Chronic hyperglycemia in diabetes mellitus, especially type 2, causes a reduction in antioxidant capacity and an increase in ROS formation. Due to membrane breakdown and PS exposure, this causes endothelial damage, compromised vascular function, and increased eryptosis [16]. Cell shrinkage and ion imbalance result from hyperosmotic stress, which occurs when extracellular osmolarity surpasses intracellular values. It frequently happens in cases of ischemia, trauma, diabetes, and dehydration [17]. Oxidative stress, cytokine release (such as IL-6 and IL-8), protein malfunction, and DNA damage are all brought on by hyperosmolarity. To combat this stress, adaptive systems, including cellular osmoregulation and ion transporters, are crucial. To maintain homeostasis, human serum typically maintains an osmolarity of 285–295 mOsm/kg [18].

Phytochemicals are natural compounds found in plants that enable them to withstand and adapt to changing environmental stresses [19]. These include alkaloids, phenolics, terpenoids, and flavonoids, which have anti-inflammatory, anti-cancer, and antioxidant properties [20]. Fruits, vegetables, whole grains, and other plants contain bioactive, non-nutritive chemical substances called phytochemicals that reduce the risk of developing chronic illnesses [21]. Many diseases have been treated alternatively using medicinal herbs. Many ailments have been treated by humans using plants and medications derived from plants [22]. They are biologically active, naturally occurring chemical elements that are present in plants and offer human beings nutritional and therapeutic advantages. They also add to the colour, perfume, and flavour of the plant while protect it from harm and illness [23].

A polyphenolic substance called chysin (5, 7 dihydroxy flavone) is obtained from species including passiflora, pelargonium, and pinaceae) [24]. It has potent hepatoprotective, anti-inflammatory, anti-diabetic, and antioxidant qualities. Chrysin strengthens antioxidant defenses and scavenges free radicals to prevent oxidative damage [25]. In cancer models, it has demonstrated effectiveness in lowering liver damage, avoiding eryptosis, and providing chemopreventive advantages [26]. High quantities may be harmful to some cell types, even though daily doses up to 3 grams are usually safe. High quantities may be harmful to some cell types, even though daily doses up to 3 grams are usually safe. [27].

MATERIALS AND METHODS

This research was carried out at the Cellular Biochemistry Research Laboratory (CBRL), Department of Biochemistry, University of Agriculture, Faisalabad.

Experimental Work

With the approval from the Directorate of Graduate Studies (DGS), University of Agriculture, Faisalabad, experimental procedures were conducted. To perform the research, different methodologies and techniques were used depending on the required purposes.

Sample Collection

The blood samples were collected from Allied Hospital, Faisalabad, Pakistan and secured in heparin tubes. and secured in heparin tubes.

Erythrocytes Isolation

Firstly, blood samples were centrifuged at 1500 revolutions per minute for 4-5 min to separate plasma. Washing of blood was done by adding 2 ml of ringer solution to the previously centrifuged sample. Then again centrifugation was carried out at 1500 revolutions per minute for 4-5 min. The supernatant was removed by using micro-pipette and erythrocytes were obtained [28].

Preparation of sample

Erythrocytes that were centrifuged at 1500 revolutions per minute for 4-5 min were treated with different concentrations of sucrose to show how eryptosis was induced with the use of sucrose. Then, a 5 mM stock

solution of chrysin was prepared, and then from this stock solution of chrysin, different concentrations such as 5, 10, and 15 uM of chrysin were used to treat these erythrocytes to show the inhibitory effect of the chrysin. 1 control and 4 treatments were implied. After that, the prepared samples were incubated for 48 hours.

Hemolysis Measurement

When incubation of both samples was completed for 48 hours then micro-centrifuged at 3000 revolutions per minute for 3-4 min. After that supernatant, containing hemoglobin, was collected, and the hemolysis percentage was calculated from this supernatant at 571 nm in a spectrophotometer [29].

Measurement of Oxidative Stress

Different antioxidants are naturally present in erythrocytes to combat ROS that are produced within them. Trapping compounds, carrier proteins, different antioxidant enzymes, and a pharmacological defense system are present in the body. Vitamins A, D, and GSH are defense molecules, while enzymes such as CAT, GPx, and SOD are defense molecules. Furthermore, a variety of phytochemicals have anti-oxidant properties and can be utilized to lessen oxidative stress in cells.

Superoxide Dismutase

SOD activity was assessed to determine the cell's antioxidant potential. Superoxide was produced from riboflavin and methionine, which either reduced NBT (a source of chromophore) or was oxidized by SOD. SOD activity at 570 nm was measured in both types of samples using an Eliza plate reader [28]. The lower degree of photo-reduction caused by NBT is correlated with SOD activity.

The Reaction Mixture of SOD

The reaction mixture components of SOD were prepared as: 15 milliliters of distilled water to dissolve 0.035 grams of triton X-100, 30 milliliters of distilled water were used to combine 0.03 grams of NBT, 0.44 grams of methionine, and 0.07 grams of riboflavin. Each of these solutions was combined in 800µL of distilled water, 100µL of NBT, 200µL of methionine, and 200µL of Triton-X, and then dissolved in 0.2M phosphate buffer [29] [30].

Assay Procedure for Superoxide Dismutase

50µL of samples and 50µL of SOD reaction mixture were put into a 96-well microtiter plate. After that, it was exposed to the UV light for fifteen minutes. After 15 minutes, mix with 25 µL of riboflavin. After that, absorbance at 570nm was measured using an ELISA plate reader [29] [30].

Glutathione Peroxidase (POD)

Glutathione peroxidase transforms H_2O_2 into H_2O and O_2. The Elisa plate reader measures the absorbance of the brown chemical that is created when oxygen and guaiacol react.

The Reaction Mixture of Glutathione Peroxidase (POD)

The POD reaction mixture was formed combining 400µL of 0.2 M phosphate buffer, 100µL of 40 mM H_2O_2, and 100µL of 20 mM Guaiacol [29].

Assay Procedure for POD

After loading 50µL of samples and 50µL of POD reaction mixture into a 96-well microtiter plate, the plate was put in an ELISA plate reader, and the absorbance at 490nm was measured [28]. Same process was used for sucrose and chrysin samples.

Catalase (CAT)

The catalase enzyme transformed H_2O_2 into H_2O and O_2, and the Elisa plate reader was used to detect absorbance.

The Reaction Mixture of CAT

The CAT reaction mixture is created by combining 100 µL of phosphate buffer with 100 µL of H_2O_2 [29].

Assay Procedure for CAT

96-well microtiter plates were loaded with 50µL of samples and 50µL of CAT reaction mixture in separate wells. The plates were then kept in an ELISA plate reader to measure absorbance at 240nm [28]. Same process was used for different concentrations of sucrose and chrysin.

Measurement of Cell Size

Eryptosis is defined by specific features like shrinkage of cell and membrane blebbing. The MCV of red blood cells was calculated by using an automated hematology analyzer [29].

Calcium Role Confirmation

To confirm the role of calcium on eryptosis, a Ca^{2+} channel blocker amlodipine was used. They treated MCV (mean cell volume) with a 1mM concentration of amlodipine and utilized a hematology analyzer to verify the contribution of Ca^{+2} in initiating eryptosis [29].

Statistical Analysis

ANOVA and Tukey's post-hoc test were used for statistical analysis. Tukey's test assisted in identifying particular differences between the control and treatment groups, while ANOVA was utilized to evaluate the overall significance between them. For accuracy and dependability, each experiment was conducted over twelve times.

RESULTS

The current research entitled as "Determination of the role of Chrysin in erythrocytes survival" was performed in CBRL, UAF. The effect of Chrysin on the hemolytic activity, antioxidants enzymatic activity, and mean cell size measurement of erythrocytes was examined in this research plan. Inhibition of eryptosis, suicidal death of erythrocytes, by using various inhibitory concentrations of chrysin. Human erythrocytes were exposed to various concentrations of chrysin and incubated for 48h to inhibit eryptosis. The characteristics indications of chrysin were observed, which involve percentage hemolysis, level of antioxidant enzymes and mean cell volume. To determine the oxidative effect on erythrocytes, enzyme assays were conducted to measure the levels of antioxidants, glutathione peroxidase, catalase, and superoxide dismutase. High levels of amlodipine with chrysin's action helped to close cationic channels, which in turn increased the suppression of eryptosis. For the treatment of eryptosis different concentrations of chrysin were used amount (5µM, 25µM, 50µM) were used.

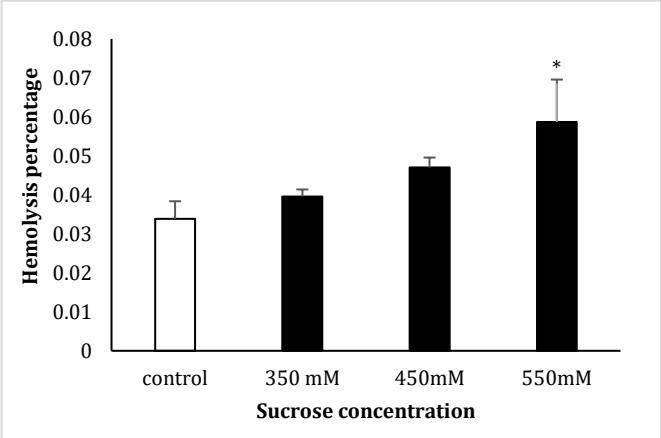
Measurement of Hemolysis

Variation in Hemolysis % due to Sucrose

Hemolysis is correlated with eryptosis. Estimation of hemolysis was done with the exposure of sucrose at different concentrations (350mM, 450 mM, 550 mM) to erythrocytes. Supernatant is collected and poured on ELISA plate and concentration of hemolysis was observed through spectrophotometer.

Fig.4.1.1

Variation in hemolysis due to sucrose

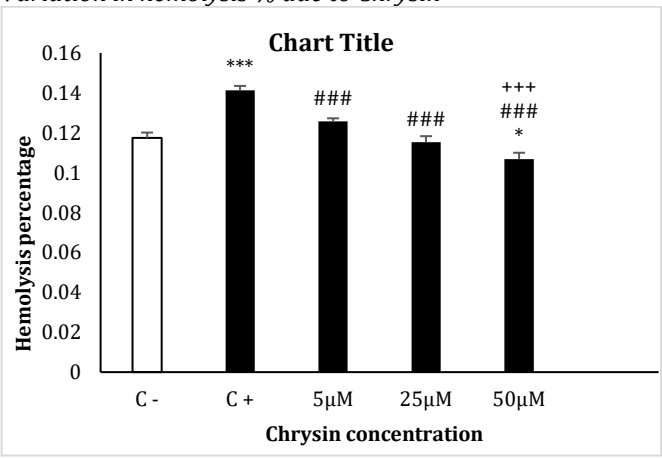


Arithmetic means ± SEM (n=12) were calculated. Erythrocytes were treated with sucrose and incubated for 48 hours. Erythrocytes in absence of sucrose (control) is shown with white bar and in the presence of sucrose (350mM, 450mM, 550mM) is shown with black bars respectively. Graph is plotted between hemolysis % and different sucrose concentrations. The variation in treated and non-treated samples were *($p<0.05$). Results showed that hemolysis percentage in sample increased with increasing sucrose concertation.

Fig.4.1.1 Illustrated a highly significant increase in hemolysis level in erythrocytes on exposure to different concentrations of sucrose 350Mm, 450Mm, 550Mm respectively with respect to control *($p<0.05$). Results showed that hemolysis percentage increases with increase in concentration of sucrose.

Fig 4.1.2

Variation in hemolysis % due to Chrysin



Arithmetic means ± SEM (n=12) were calculated. Erythrocytes were treated with Chrysin and incubated for 48 hours. Erythrocytes in absence of sucrose (control -) is shown with white bar and in the presence of sucrose 550 mM (control+) is shown with grey bar and erythrocytes in the presence of sucrose and chrysin (5µM, 25µM and 50µM) is shown with black bars respectively. Graph is plotted between hemolysis % and different Chrysin concentrations. The obvious distinction between treated and non-treated samples were showed ***($p<0.001$), *($p<0.05$) and among treatments were showed ###($p<0.001$) and+++ ($p<0.001$).

Fig.4.1.2. Illustrated highly significant decrease in hemolysis level in erythrocytes on exposure to different concentrations of chrysin (5µM, 25µM and 50µM) respectively with respect to control ***($p<0.001$), *($p<0.05$) and result found between treatments were shows ###($p<0.001$), ###($p<0.001$), ###($p<0.001$) and+++ ($p<0.001$). Results showed that hemolysis % decreases with increasing in concentration of Chrysin.

This study supports previous research showing that polyphenols and flavonoids in Chrysin have cytoprotective and antioxidant properties [31].

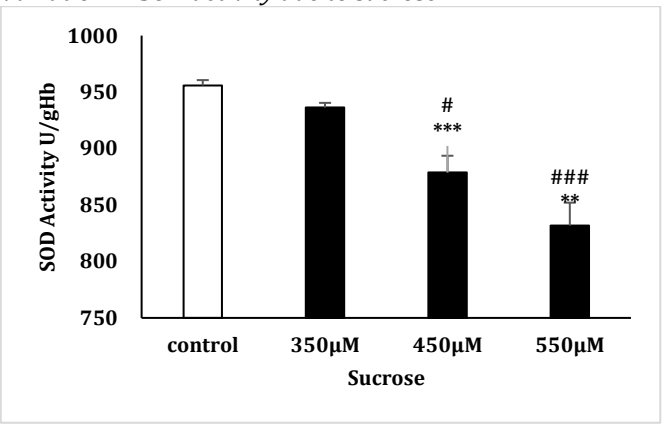
Oxidative Stress Measurement

To determine consequence of sucrose against oxidative stress, antioxidants defense was evaluated by reader of ELISA plate.

Superoxide Dismutase

Fig.4.2.1

Variation in SOD activity due to sucrose

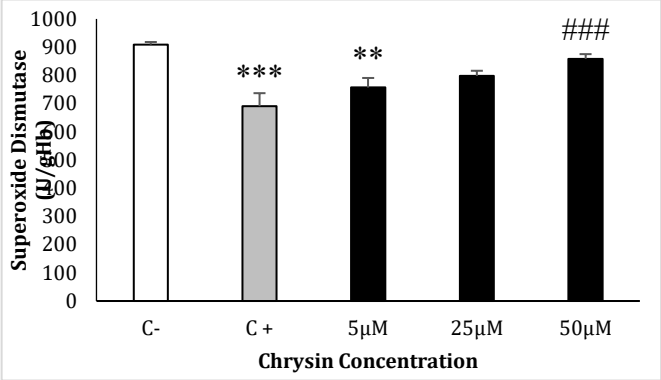


Arithmetic means ±SEM (n=12) were calculated. Erythrocytes were treated with sucrose and incubated for 48 hours. Erythrocytes in the absence of sucrose (control) is shown with white bar and in the presence of sucrose (350mM, 450mM, 550mM) is shown with black bars respectively. Graph is plotted between SOD activity different sucrose concentrations. The obvious distinction between control and treatment in enzyme activity showed when ***($p<0.001$), ***($p<0.001$) and result found between treatments were shows #($p<0.05$) and ###($p<0.001$). Results showed that decrease in SOD activity due to increasing sucrose concentration.

Fig.4.2.1 Illustrated a highly significant decrease in superoxide dismutase activity in erythrocytes on exposure to different concentrations of sucrose 350Mm, 450Mm, 550Mm respectively with respect to control ***($p<0.001$), ***($p<0.001$) and result found between treatments were shows #($p<0.05$) and ###($p<0.001$). Results showed that decrease in superoxide dismutase activity by increased concentration of sucrose.

Fig. 4.2.1.1

Variation in SOD due to Chrysin.



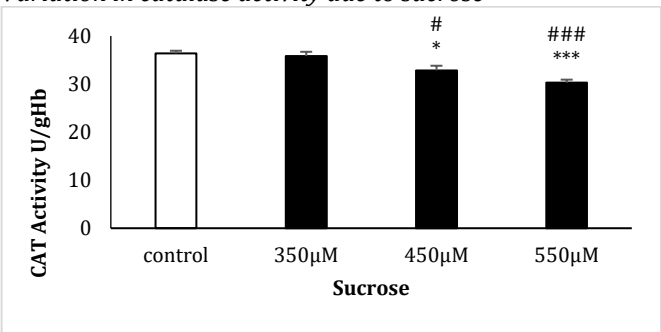
Arithmetic means ± SEM (n=12) were calculated. Erythrocytes were treated with sucrose and Chrysin and incubated for 48 hours. Erythrocytes in the absence of sucrose (control -) is shown with white bar and in the presence of sucrose 5.5mM (control+) is shown with grey bar and erythrocytes in the presence of sucrose and chrysin (5µM,25µM and 50µM) is shown with black bars respectively. Graph is plotted between superoxide dismutase activity and different chrysin concentrations. The obvious distinction between control and treatment in enzyme activity were showed ***(p<0.001), **(p<0.01) and among treatments were showed $^{\#\#\#}$(p<0.001). The results showed that there was increase in superoxide dismutase activity due to increase in chrysin concentration.

Fig.4.2.1.1. Illustrated a highly significant increase in superoxide dismutase activity in erythrocytes on exposure to different concentrations of chrysin (5µM, 25µM and 50µM) respectively with respect to control ***(p<0.001) **(p<0.01) and among treatments were shows $^{\#\#\#}$(p<0.001). Results showed that superoxide dismutase activity increases with increasing in concentration of chrysin. My result of my research is similar to that of [32], [33] and [34].

Catalase

Fig.4.2.2

Variation in catalase activity due to sucrose

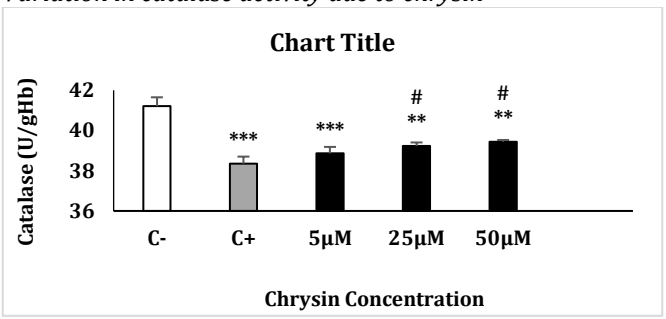


Arithmetic means ±SEM (n=12) were calculated. Erythrocytes treatment with sucrose and incubated for 48 hours. Erythrocytes in absence of sucrose (control) is shown with white bar and in the presence of sucrose (350mM, 450mM, 550mM) is shown with black bars, respectively. Graph is plotted between CAT activity and different sucrose concentrations. The obvious distinction between control and treatment in enzyme activity were shows *(p<0.05), ***(p<0.001) among treatments were showed $^{\#}$(p<0.05) and $^{\#\#\#}$(p<0.001). Results showed that there was decrease in catalase activity due to increase sucrose concentration.

Fig.4.2.2. Illustrated a highly significant decrease in catalase activity in erythrocytes on exposure to different concentrations of sucrose 350Mm, 450Mm, 550Mm respectively with respect to control were shows *(p<0.05) ***(p<0.001) among treatments were showed $^{\#}$(p<0.05) and $^{\#\#\#}$(p<0.001). Results showed that there was decrease in catalase activity by increasing concentration of sucrose.

Fig.4.2.2.1

Variation in catalase activity due to chrysin



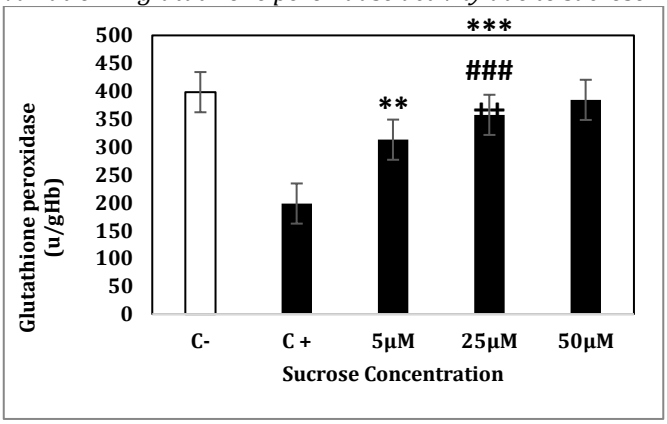
Arithmetic means ± SEM (n=12) were calculated. Erythrocytes were treated with sucrose and chrysin and incubated for 48 hours. Erythrocytes in absence of sucrose (control -) are shown with a white bar, and in the presence of sucrose 5.5mM (control+) are shown with grey bar Erythrocytes in the presence of sucrose and chrysin (5µM, 25µM, and 50µM) are shown with black bars, respectively. A graph is plotted between catalase activity and different chrysin concentrations. The obvious distinction between control and treatment in enzyme activity were showed ***(p<0.001) ***(p<0.001), **(p<0.01), **(p<0.01), and among treatment were showed $^{\#}$(p<0.05) and $^{\#}$(p<0.05). The results showed that there was an increase in catalase activity due to an increase in chrysin concentration.

Fig.4.2.2.1. Illustrated a highly significant increase in catalase activity in erythrocytes on exposure to different concentrations of chrysin (5µM, 25µM and 50µM) respectively with respect to control ***(p<0.001) ***(p<0.001), **(p<0.01), **(p<0.01) and among treatment were showed $^{\#}$(p<0.05) and $^{\#}$(p<0.05). Results showed that catalase activity increases with increasing in concentration of chrysin. Similar results were showed [35], [36] and [32] in their studies.

Glutathione Peroxidase

Fig.4.2.3

variation in glutathione peroxidase activity due to sucrose

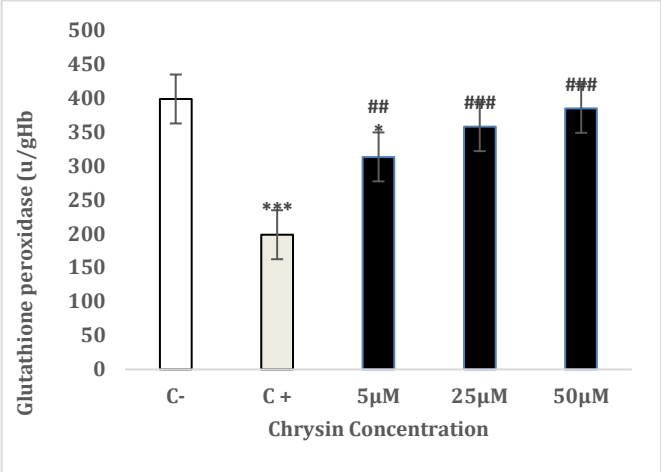


Arithmetic means ±SEM (n=12) were calculated. Erythrocytes were treated with sucrose and incubated for 48 hours. Erythrocytes in absence of sucrose (control) are shown with a white bar, and in the presence of sucrose (350mM, 450mM, 550mM) are shown with black bars, respectively. A graph is plotted between glutathione peroxidase and different sucrose concentrations. The obvious distinction between control and treatment in enzyme activity showed when **(p<0.01), ***(p<0.001), and result found among treatments show ### (p<0.001) and ++(p<0.01). Results showed that there was a decrease in glutathione peroxidase activity due to increasing sucrose concentration.

Fig.4.2.3. Illustrated a highly significant decrease in glutathione peroxidase activity in erythrocytes on exposure to different concentrations of sucrose, 350Mm, 450Mm, 550Mm respectively concerning control **(p<0.01), ***(p<0.001) and the results found among treatments shows### (p<0.001) and ++(p<0.01). Results showed that there was a decrease in glutathione peroxidase activity with increased concentration of sucrose.

Figure 4.2.3.1

Variation in glutathione activity due to Chrysin



Arithmetic means ± SEM (n=12) were calculated. Erythrocytes were treated with sucrose and Chrysin and incubated for 48 hours. Erythrocytes in absence of sucrose (control -) are shown with white bar, and in the presence of sucrose 550 mM (control+) are shown with grey bar Erythrocytes in the presence of sucrose and Chrysin (5µM, 25µM, and 50µM) are shown with black bars, respectively. A graph is plotted between glutathione peroxidase activity and different chrysin concentrations. The obvious distinction between control and treatment in enzyme activity was shows ***(p<0.001), *(p<0.05), and among treatments were showed ##(p<0.01), ###(p<0.001) and ###(p<0.001). Results showed that there was increase in glutathione peroxidase activity due to increasing in chrysin concentration.

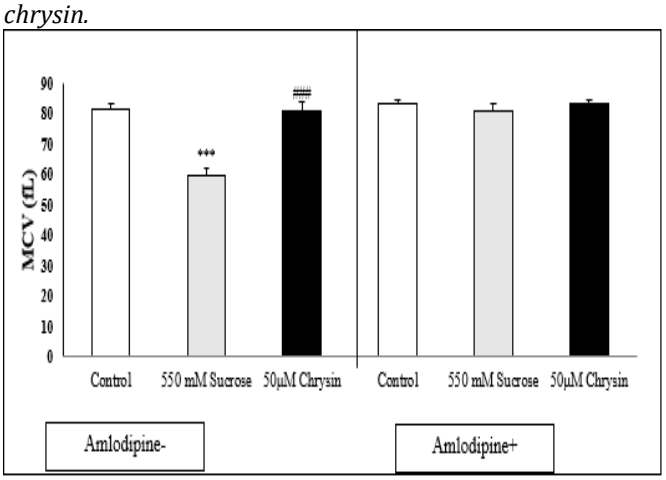
Fig.4.2.3.1. Illustrated a highly significant increase in glutathione dismutase activity in erythrocytes on exposure to different concentrations of chrysin (5µM, 25µM and 50µM) respectively with respect to control were shows***(p<0.001), *(p<0.05) and among treatments were shows ##(p<0.01), ###(p<0.001) and ###(p<0.001).

Results showed that glutathione peroxidase activity increases with increasing concentration of chrysin. The almost same results were seen [37], [38] and [39].

Variation in Ca^{2+} activity of amlodipine due to sucrose and chrysin

Figure 4.3

Variation in Ca^{2+} activity of amlodipine due to sucrose and chrysin.



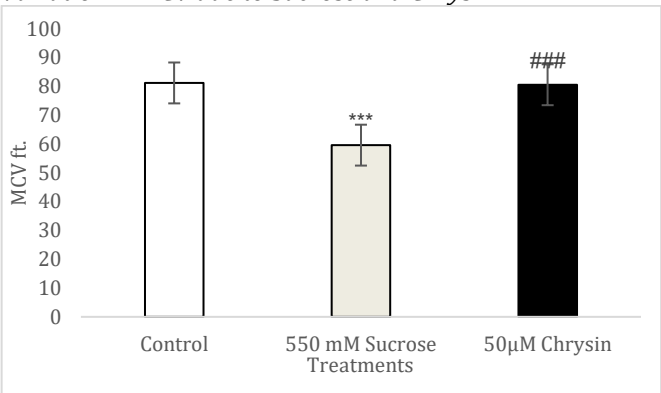
Arithmetic means ± SEM (n=12). Erythrocytes were subjected to treatment with 550 mM sucrose and 50 µM Chrysin and then incubated for 24 hours. A graph was generated with sucrose and chrysin concentrations on the X-axis and mean corpuscular volume (MCV) on the Y-axis. In the graph, Control was represented by white bars, erythrocytes treated with chrysin were represented by gray bars, and erythrocytes treated with both sucrose and chrysin were represented by black bars. Statistical analysis indicated significant differences between the treated and untreated samples, denoted by *** ($p < 0.001$), as well as among the sucrose and chrysin treated samples, denoted by ### ($p < 0.001$), specifically in amlodipine negative samples.

The results revealed that cell volume decreased when treated with sucrose, while chrysin treatment led to an increase in cell volume in the absence of amlodipine. However, there was no significant difference in MCV for amlodipine-treated samples. These findings demonstrated that the activity of blocking Ca^{2+} ion entry increased in the presence of amlodipine compared to the absence of amlodipine, specifically when erythrocytes were exposed to sucrose and chrysin under certain conditions. It was previously observed that sucrose acted as a stimulator, increasing calcium entry, and similar results were found with significant changes in calcium blocking activity. The calcium blocking activity was enhanced in treated erythrocytes due to the antioxidant effects of chrysin. The scavenging of free radicals induced by chrysin contributed to the increased Ca^{2+} blocking activity and free radical scavenging activity. These results imply that amlodipine effectively blocked Ca^{2+} channels and Chrysin was unable to increase intracellular Ca^{2+} level which was the cause of cell shrinkage and eryptosis. Similar results of blockage of Ca^{2+} channels by amlodipine were reported by [40], [41] and [42].

MCV

Figure 4.4.

Variation in MCV due to Sucrose and Chrysin



Arithmetic means ± SEM (n = 12) were calculated. Erythrocytes were treated with 5mM sucrose and 50µM chrysin, and incubated for 1 day. A graph was plotted between sucrose and chrysin and MCV. The untreated erythrocytes are shown in white bar and sucrose treated erythrocytes with gray bar and Chrysin treated with black bar. The remarkable variation between treated and non-treated samples were showed ***($P<0.001$) and among sucrose and chrysin treated samples were showed ###($P<0.001$). Results showed that cell volume decreases on treatment with sucrose while treatment with chrysin increases cell volume. Results demonstrated that treatment with sucrose triggers shrinkage of cell leading to eryptosis while treatment with chrysin prevents erythrocytes from shrinkage thus inhibits eryptosis. Similar evidences of cytoprotective property of Chrysin were discussed by [43] and [44].

DISCUSSIONS AND CONCLUSION

This study provides strong evidence that Chrysin, a natural compound in Passiflora, Pelargonium, and Pinaceae can significantly protects erythrocytes from glucose induced eryptosis. Induction of glucose to erythrocytes significantly disrupts natural redox balance by reducing antioxidant enzymes such as SOD, GPx and CAT, increases hemolytic percentage, disrupts morphology of cell causing cell shrinkage and increased calcium influx. However, treatment with Chrysin significantly counters it and put forward a strong anti-oxidant and cytoprotective role. Chrysin restored the redox balance and stabilizes the cell membrane [31].

Oxidative stress happens when the production of reactive oxygen species overcomes the cell's antioxidant defenses system. This leads to the structural and functional damage of the cells. In our study, erythrocytes when treated with different dose of sucrose, exhibited high levels of hemolysis percentage, indicating cell membrane disruption under oxidative stress. While, the oxidative damage was countered and mitigated by Chrysin in a dose dependent manner. These results confirm the role of Chrysin in mitigating glucose induced eryptosis. These results may also be attributed to the presence of active constituents like Flavonoids, which neutralize ROS and support membrane integrity. Although it is the first study conducted on human erythrocytes to assess the role of Chrysin in countering glucose induced eryptosis but this observation is consistent with other studies that display antioxidant and membrane-stabilizing properties [32] and [44].

Additionally, our results showed that the antioxidant enzymes— GPX, SOD, and CAT were significantly reduced under glucose stress, but were restored close to normal levels following Chrysin treatment. This suggests that Chrysin 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 Chrysin helps prevent the characteristic cell shrinkage seen in eryptosis as discussed in [43] and [44].

Another important aspect of this research was to verify role of calcium in the triggering of eryptosis induced by glucose. When a calcium channel blocker, amlodipine, was introduced to erythrocytes, results indicated that there was no significant decrease in the MCV of cells. This outcome showed that amlodipine effectively blocked the calcium channels that were the target of glucose thus no increase in intracellular Ca^{2+} level happens that was the cause of the onset of eryptosis.

In nutshell, the results indicate that Chrysin is a potent phytochemical that protects erythrocytes from the oxidative damage. It exhibits both antioxidant and cytoprotective properties, thus counters ROS, maintain redox balance, maintain integrity and morphology of cell and enhances the antioxidant activity of natural antioxidant enzymes. This makes Chrysin a promising compound for treating disorders related to oxidative stress. Although our in vitro data are encouraging, yet future studies need to be focused on determining the molecular pathways being involved, testing the efficacy of Chrysin in in-vivo settings and determining pharmacokinetics.

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