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Allicin based inhibition of eryptosis: A novel therapeutic approach against hyperosmolarity triggered anemia

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Abstract: **Background**: Hyperosmotic stress is often overlooked in various clinical conditions like diabetes and may lead to anemia. Erythrocytes are very sensitive cells in body circulation and are affected by very minute changes in the environment. Eryptosis (suicidal death of erythrocytes) is mainly characterized by phosphatidylserine exposure, cell shrinkage, blabbing and is triggered by hyperosmolarity, oxidative stress, energy depletion, xenobiotic exposure and activation of various kinases. **Objective**: This study was planned to evaluate the eryptotic effects of hyperosmolarity by stimulating p38 MAPK and enhanced ROS generation and allicin's anti-hemolytic, antioxidant, p38MAPK inhibitory and anti-eryptotic potential. **Methods**: Effects of allicin on erythrocytes facing hyperosmolarity were confirmed by measuring enzyme activity (SOD, GPx and CAT), ROS level, eryptotic marker (change in cell size and phosphatidylserine exposure) and underlying mechanisms of eryptosis (role of Ca^{2+} in eryptosis and p38 MAPK activation) were confirmed. To measure the cytotoxic effects of allicin hemolysis % was measured. To check statistical significance of data ANOVA was used. **Results**: Results confirmed that allicin treatment enhances SOD, GPx and CAT activity, reduces ROS level, p38 MAPK activation and Ca^{2+} effects, positively affects cell size (normal range) and phosphatidylserine exposure (reduced) and reduces hemolysis % in erythrocytes facing hyperosmolarity. **Conclusion**: It is concluded from this study that hyperosmolarity is the main stimulator of eryptosis and leads to anemia in various clinical conditions, while allicin treatment helps to increase erythrocyte survival by inhibiting and attenuating various pathways and reducing eryptpotic rate. Allicin's anti-eryptotic and cytoprotective potential gives foundation for future clinical research targeting hyperosmolarity triggered anemia.

Keywords: Allicin; Eryptosis; Hyperosmolarity; Oxidative stress; Phosphatidylserine; p38MAPK; ROS

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INTRODUCTION

Hyperosmolarity is a condition in which blood is more viscous than normal due to high concentration of solutes like glucose, sodium and other substances. For body cells, maintenance of constant volume is of great importance for survival and proper functioning (Argyropoulos *et al.*, 2016). Diabetes, chronic kidney disease and different infections could lead to lasting hyperosmotic state (in which serum glucose> 600mg/dl and serum osmolarity > 320mOsm) (Pasquel *et al.*, 2020; Hassan *et al.,* 2022; Mohajan and Mohajan, 2023). Eryptosis is the suicidal cell death of erythrocytes triggered by hyperosmolarity, high oxidative stress energy depletion (Klarl *et al.*, 2006; Gatidis *et al.*, 2011), xenobiotic exposure, ceramide production and dysregulation of several kinases (Alghareeb and Alfhili, 2023). Erythrocyte shrinkage, plasma membrane blebbing and scrambling with phosphatidylserine translocation to the outer leaflet of erythrocyte's surface are the hallmarks of suicidal erythrocyte death (Repsold and Joubert, 2018). The older an erythrocyte is, the more susceptible it is to eryptosis (Ghashghaeinia *et al.*, 2012).

Hyperosmolarity increases reactive oxygen species (ROS) generation (Zheng *et al.*, 2014) and activates phospholipase A2, which further stimulates platelet activation factor and leads to sphingomyelin breakdown and formation of ceramide (Pretorius *et al.*, 2014). Ceramide production stimulates p38 mitogen activated protein kinase (p38MAPK) (Chen *et al.*, 2008). The p38 MAPK participates in the regulation of the cell volume (Cowan and Storey, 2003; Gatidis *et al.*, 2011). High oxidative stress and P-p38 MAPK stimulate Ca^{2+} channels thus regulating Ca^{2+} entry inside the cell (Li *et al.*, 2021; Föller *et al.*, 2013). An increase in cytosolic Ca^{2+} activity causes both shrinkage of cells and membrane scrambling (phosphatidylserine exposure) (markers of eryptosis) (Lang *et al.,* 2012). Thus, hyperosmotic shock is likely involved in triggering various mechanisms involved in the suicidal cell death of erythrocytes (Mikhailov *et al.,* 2005).

Medicinal plants are the primary source of medicine for the treatment of human diseases in many rural areas of developing countries. The medicinal value of the plant is due to the presence of various bioactive chemical constituents such as alkaloids, tannins, flavonoids and phenolic compounds (Sultana *et al.,* 2012). The present study explores allicin's (a flavonoid) anti-eryptotic potential in hyperosmotically stressed erythrocytes. Garlic has benefits for lowering blood pressure, ageing and infections (Powolny and Singh, 2008). On the other hand, when a clove is chopped or crushed, alliin and alliinase

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combine to generate allicin, which is a mediator of inflammation and gives garlic its strong flavor and antibacterial qualities. Garlic has also been suggested to prevent the development of colorectal tumors, lower blood pressure and cholesterol, inhibit clotting and have wide antioxidant activity to protect against damage from free radicals (Alpers, 2009). Research showed that allicin inhibits p38MAPK, a stimulator or eryptosis (Li *et al.*, 2015).

Eryptosis is silent driver of anemia, especially in diabetic patients. Eryptosis is a physiological process, but during stress, its rate accelerates and may lead to anemia, which further complicates the diabetic situation through microvascular injury, thrombosis formation and microcirculation blockage. Anemia further induces tissue hypoxia, delays wound healing, enhance ischemic vulnerability. Anemia during Diabetes mellitus not only complicates the situation itself but also leads to cardiovascular, renal and retinal complications. Conventional therapies are focused on the production of erythrocytes rather than preventing their premature death. Modulating eryptosis to treat hyperosmolarity trigger anemia would be a novel therapeutic intervention.

This study was designed to evaluate the eryptotic effects of hyperosmolarity by stimulating p38MAPK and enhanced ROS generation and allicin's potential to inhibit eryptosis by inhibiting p38MAPK expression and free radicals production.

MATERIALS AND METHODS

Workplace

All experimental work was conducted at the Cellular Biochemistry Research Laboratory, Department of Biochemistry, UAF and Hormones Lab, Lahore. The study was conducted after the approval by the Institutional Bioethics Committee (IBC) and the Directorate of Graduate Studies (DGS) (approval no: 492/ Date: 01-02-2024), University of Agriculture, Faisalabad, Pakistan.

Erythrocytes, chemicals and solutions

Screened blood samples were obtained from blood bank of Allied Hospital, Faisalabad, Pakistan. Erythrocytes were isolated from a whole blood sample by using the reported protocol (Fink *et al.*, 2018). Blood samples were centrifuged at 1500rpm for 15 min, after centrifugation erythrocytes are obtained by removing plasma and buffy coat (WBCs, platelets) from the samples. Erythrocytes incubation was done at various hematocrits (0.4% and 0.40%) according to experimentation requirements. For incubation, erythrocytes were suspended in Ringer's solution containing (in mM) 5 KCl, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 125 NaCl, 1 $CaCl_2$, 5 glucose, 1 $MgSO_4$, pH 7.4 at 37°C for 24-48 hours. Sucrose (550mM) was used to induce hyperosmotic environment for erythrocytes (Gatidis *et al.*, 2011). Erythrocytes were exposed to various concentrations of allicin (25, 50 and 75µM) to confirm its anti- eryptotic potential under isotonic and hypertonic conditions.

Osmolarity measurement

Osmolarity of samples was measured by using osmometer (WESCOR). Erythrocytes were suspended in Ringer's solution along with 550 mM sucrose (Gatidis *et al.*, 2011) and various concentrations of allicin before measuring the osmolarity of samples.

Measurement of oxidative stress

Hyperosmolarity induced oxidative stress and allicin's antioxidant potential was confirmed by performing the antioxidant enzyme assays.

Superoxide dismutase (SOD) assay

Erythrocytes were incubated at a hematocrit of 0.4% along with allicin (25, 50, 75µM) in isotonic and hypertonic conditions for 48 hours at 37ºC. SOD activity was measured by following the reported protocol (Mukhtar *et al.*, 2022). Preparation of SOD reaction mixture required (NBT 0.015 g in 17.5 mL H_2O, methionine 0.222 g in 15mL H_2O, riboflavin 0.0132 g in 17.5 mL H_2O, Triton-X 0.0375 mL in 17.5 mL H_2O and buffer 0.2 M). After preparing all these solutions, the reaction mixture was prepared by mixing NBT 100 µ l, distilled water 800 µ l, Triton-X 200 µ l and phosphate buffer 200 µl. To measure the SOD activity, 50 µl samples, 50 µl reaction mixture and 25 µl riboflavin were added to a 96-well plate and the plate was kept under an ultraviolet radiation lamp for 10 minutes. An ELISA plate reader (BioTek- PYI) was used to measure absorbance in a 96-well plate at 560nm (Mukhtar *et al.*, 2022).

Glutathione peroxidase (GPx) assay

Erythrocytes were incubated at a hematocrit of 0.4% along with allicin (25, 50, 75µM) in isotonic and hypertonic conditions for 48 hours at 37ºC. GPx levels were measured by following the reported protocol (Mukhtar *et al.*, 2022). Preparation of GPx reaction mixture required solutions [H_2O_2 40 mM (100 µl), guaiacol 20 mM (100 µl) and phosphate buffer (400 µl)]. To measure the GPx activity, 50 µl samples and 50 µl reaction mixture were added to a 96-well plate. An ELISA plate reader (BioTek- PYI) was used to take readings of a 96-well plate at 470nm (Sattar *et al.*, 2022).

Catalase (CAT) assay

Erythrocytes were incubated at a hematocrit of 0.4% along with allicin (25, 50, 75µM) in isotonic and hypertonic conditions for 48 hours at 37ºC. Catalase activity was measured by following the reported protocol (Mukhtar *et al.*, 2022). Preparation of the catalase reaction mixture required solutions of H_2O_2 (5.9 mM; 1000 µl) and phosphate buffer (0.2 M; 1000 µl). To measure the catalase activity, 50 µl samples and 50 µl reaction mixture were added to a 96-well plate. An

ELISA plate reader (BioTek- PYI) was used to measure absorbance in a 96-well plate at 240nm. (Sattar *et al.*, 2022).

Measurement of ROS

ROS measurement was done by following the protocol given by (Hayashi *et al.*, 2007). Samples were prepared by incubating erythrocytes at a hematocrit of 0.4% along with allicin (25, 50, 75µM) in isotonic and hypertonic conditions for 48 hours at 37ºC. For this experiment 2 solutions were made: in solution 1 (S1) N, N-diethyl-para-phenylendiamine (DEPPD) was dissolved in 0.1 molar sodium acetate buffer of 4.8pH, final concentration obtained was 100µg/mL, S1 solution was a chromogen (a solution that gives color upon reaction), S2 solution was made by dissolving ferrous sulfate in 0.1 M sodium acetate buffer of pH 4.8, R2 solution contains a transition metal ion, end molarity of that solution was set to 4.37µM.

The reaction mixture was prepared by mixing the S1 and S2 solutions in a 1:25 ratio prior to use. 50µl of sample, along with 50 µl of reaction mixture, was added to 96-well plates. After one minute incubation at 37ºC, the sample, which was contained in a 96-well plate, was placed in an ELISA plate reader (BioTek- PYI) and absorbance was checked at 505nm.

Cell size measurement

In addition to forward scatter, change in erythrocytes' volume (a marker of suicidal death of erythrocytes) was investigated by measuring the mean cell volume, using an automated hematology analyzer (SYSMEX XP-100) (Rana *et al.*, 2019). Erythrocytes were incubated at a hematocrit of 0.4% along with allicin (75µM) in isotonic and hypertonic conditions for 48 hours at 37ºC and a change in MCV was checked.

Role of Ca^{2+} in the stimulation of eryptosis

To confirm the role of Ca^{2+} in the stimulation of eryptosis, erythrocytes were exposed to 10µM amlodipine (a calcium channel blocker) (Khan *et al.*, 2019), along with allicin (75µM) at a hematocrit of 0.4% in isotonic and hypertonic conditions for 48 hours at 37ºC. The inhibition of eryptosis in amlodipine treated cells was confirmed through MCV (change in cell volume) measurement by using hematology analyzer (SYSMEX XP-100) (Sattar *et al.*, 2022).

Measurement of P-p38 MAPK

For P-p38 MAPK detection, P-p38MAPK (Thr180/Tyr182) In-Cell ELISA Kit (ab126425) was used. The experiment was performed according to the given instructions of the manufacturer. After incubation time protein extract was incubated in ELISA plate immobilized with specific antibodies and followed by incubation with a secondary antibody conjugated with a horseradish peroxidase for detection through a micro-plate reader. Percentage reduction was calculated by using formula: percentage reduction= [(Control value-Treatment value)/ Control value] ×100.

Measurement of phosphatidylserine exposure

Erythrocytes were incubated at a hematocrit of 0.4% along with allicin (75µM) in isotonic and hypertonic conditions for 48 hours at 37ºC. Phosphatidylserine was measured by using a human phosphatidylserine ELISA kit (Elabscience, Beijing, People's Republic of China) according to the manufacturer's instructions. An ELISA plate reader (BioTek- PYI) was used to measure absorbance at 450mm wavelength.

Cytotoxicity measurement

For cytotoxicity evaluation, hemolysis % was measured. Erythrocytes were incubated at a hematocrit of 0.4% along with allicin (25, 50, 75µM) in isotonic and hypertonic conditions. After 48 hours of incubation at 37°C, samples were centrifuged 3 times at 3000rpm for 3 minutes. After centrifugation supernatant of samples was collected in cuvette and hemoglobin levels were measured at 405nm wavelength by using spectrophotometer (WELab) (Sattar *et al.*, 2022).

Statistical analysis

Statistical analysis was done by using GraphPad Prism software. All results were expressed ± standard error mean (SEM). One-way ANOVA was used for multiple comparisons. A *p-value* less than 0.05 were considered statistically significant.

RESULTS

Data obtained in this study are presented in figures prepared with mean ± SEM, which indicate their statistical significance. Hyperosmolarity was induced by using 550mM sucrose (Gatidis *et al.,* 2011). Results obtained confirmed the hyperosmolarity in both sucrose and allicin treated samples (Table 1). Allicin treatment exhibits very negligible effects on osmolarity.

Hyperosmolarity stimulates ROS generation and elevated ROS levels enhance the underlying mechanisms of eryptosis. The effects of possible oxidation induced by hyperosmolarity and the antioxidant effects of allicin on erythrocytes were investigated by enzyme assay. Fig. 1A illustrates allicin's effect on SOD activity of erythrocytes under hypertonic conditions in comparison to isotonic control after 48 hours of incubation. Result showed that hyperosmolarity decreases the SOD activity. Erythrocytes incubated in hypertonic conditions (facing osmolarity between 339±2.9mOsm/L) showed decrease in SOD activity. Allicin treatment enhances the SOD activity. Allicin treated erythrocytes showed maximum SOD activity at 75µM concentration. Fig. 1B, showed that hyperosmolarity decreases the GPx activity in erythrocytes. Erythrocytes incubated in hypertonic solution (facing osmolarity between 314±3.7mOsm/L)

showed decreased GPx activity. Allicin treated erythrocytes showed maximum GPx activity at 75µM concentration. Fig. 1C illustrates that hyperosmolarity decreases the CAT activity. Erythrocytes facing osmolarity between 339±3.7mOsm/L showed decrease in CAT activity. Allicin treated erythrocytes showed maximum CAT activity at 75µM concentration. Fig. 2 showed increased ROS generation in erythrocytes facing hyperosmolarity. Allicin treatment decreases ROS generation, respectively (25, 50, 75µM).

Eryptotic effect of hyperosmolarity was confirmed by measuring the change in erythrocyte volume (MCV) and phosphatidylserine exposure. Results illustrate that erythrocytes facing hyperosmolarity resulted in a significant decrease in MCV, possibly due to cell shrinkage. Allicin treatment (25, 50, 75µM), respectively, showed a shift toward normal MCV, exhibiting its involvement in inhibiting the change of erythrocyte volume (Fig. 3A). Results (Fig. 3B) reveal that allicin showed anti-apoptotic potential and somehow attenuates phosphatidylserine exposure in erythrocytes facing a hyperosmotic environment. Results confirmed that hyperosmolarity directly affects the phosphatidylserine exposure.

Confirmation of underlying mechanisms was examined by confirming the p38MAPK activity and role of Ca^{2+} in eryptosis. Role of Ca^{2+} in erythrocytes' shrinkage was confirmed by incubating eryptosis both in isotonic and hypertonic conditions, in the presence and absence of amlodipine (a calcium channel blocker). Results confirm Ca^{2+} involvement in cell shrinkage (eryptotic marker) as amlodipine-treated erythrocytes showed a very slight change in cell size in comparison to amlodipine-un-treated erythrocytes (showed shrinkage) under hypertonic conditions. Allicin treated samples under hypertonic conditions showed slight shift towards healthy range of cell size while allicin+Amlodipine samples showed combined positive effect on cell size (normal range) (Fig. 4A). Results (Fig. 4B) confirmed that hyperosmolarity stimulates p38MAPK acivation while allicin treatment 25 µM, 50 µM, 75µM reduces its activation by 2.8%, 3.9% and 8.8% respectively in erythrocytes in comparison to hypertonic control. Treatment with p38MAPK synthetic inhibitor (SB20358) (serving as positive control) significantly inhibits p38 MAPK activation and confirms pathway specificity.

The cytotoxic effects of hyperosmolarity and allicin were confirmed through hemolysis %. Results from Figure 5 indicate erythrocytes facing hyperosmolarity (550mM Sucrose exposure/ osmolarity ranges between 319-341mOsm/L) showed more hemolysis compared to those facing isotonic conditions. However, basel level of hemolysis was observed in erythrocytes facing isotonic environment due to 48h *in-vitro* incubation. Results showed decrease in hemolysis % upon allicin treatment.

DISCUSSION

Hyperosmolarity is a clinical condition observed in various infectious diseases and diabetes, where erythrocytes are continuously facing hyperosmotic and oxidative stress (Mohajan and Mohajan, 2023). As highlighted in introduction, these stresses are the major trigger of eryptosis via p38MAPK activation, ROS generation, or Ca^{2+} influx. This research work was planned to confirm allicin's ability to counteract hyperosmolarity triggered eryptosis by regulating these interconnected pathways. Studies confirmed hyperosmolarity induction upon 550mM sucrose treatment (Gatidis *et al.*, 2011). Doses of allicin (25, 50, 75µM) used have already been reported to reduce oxidative stress and inhibit p38MAPK in nucleated cells (Li *et al.*, 2015).

Findings of this study confirmed that erythrocytes facing hyperosmolarity showed a significant increase in antioxidant enzyme activity (SOD, GPx, CAT), ROS generation, activation of p38MAPK, decrease in cell volume (shrinkage; an apoptotic marker), increased PS exposure, Ca^{2+} dependent decrease in cell volume and increased hemolysis. While allicin treatment (dose dependently) enhances anti-oxidant enzymes activity, decreases ROS levels, attenuates activation of p38 MAPK, slightly shifts erythrocyte volume towards the normal range, reduces PS exposure, reduces the Ca^{2+} dependent effects of cell shrinkage and significantly lowers the hemolysis %.

Increased reactive oxygen species (ROS) production is one of the main fetal side effects of hyperosmolarity (Deng *et al.*, 2015). In every biological system, antioxidants are the enzymes that have the ability to scavenge and clear out free radicals generated during oxidation (Riaz *et al.*, 2017). Superoxide dismutase (SOD) is an antioxidant enzyme with ability to diminish O_2 based free radicals, enhanced free radical production leads to reduced SOD activity (Sattar *et al.*, 2022). Another antioxidant, catalase (CAT), plays a vital role in the direct decomposition of H_2O_2 into H_2O and O_2 (Mukhtar *et al.*, 2022). Enhanced H_2O_2 production leads to reduced CAT activity (Mladenov *et al.*, 2015). While glutathione peroxidase (GPx) uses glutathione to reduce lipid peroxides and H_2O_2 into H_2O and alcohol to prevent cells from free radical damage (Sattar *et al.*, 2022). Various studies confirmed that allicin helps to enhance the activity of SOD, GPx and CAT in nucleated cells (Yuan *et al.,* 2025; Jiang *et al*., 2025; Ke *et al*., 2024; Sun *et al.*, 2024; Jin *et al*., 2024; Tang *et al.,* 2024). Previous studies have confirmed that allicin reduces oxidative stress, ROS production and hydrogen peroxide level; indicate that allicin enhances antioxidant levels in rat cardiomyoblast cells (Chan *et al.*, 2014).

Table 1: Osmolarity of allicin treated erythrocytes (screened) sample

Osmolarity range (mOsm/L)		
Samples	Isotonic solution	Hypertonic solution
Control	279±1.6	341±3.1
Allicin	277±4.3	338±9.4

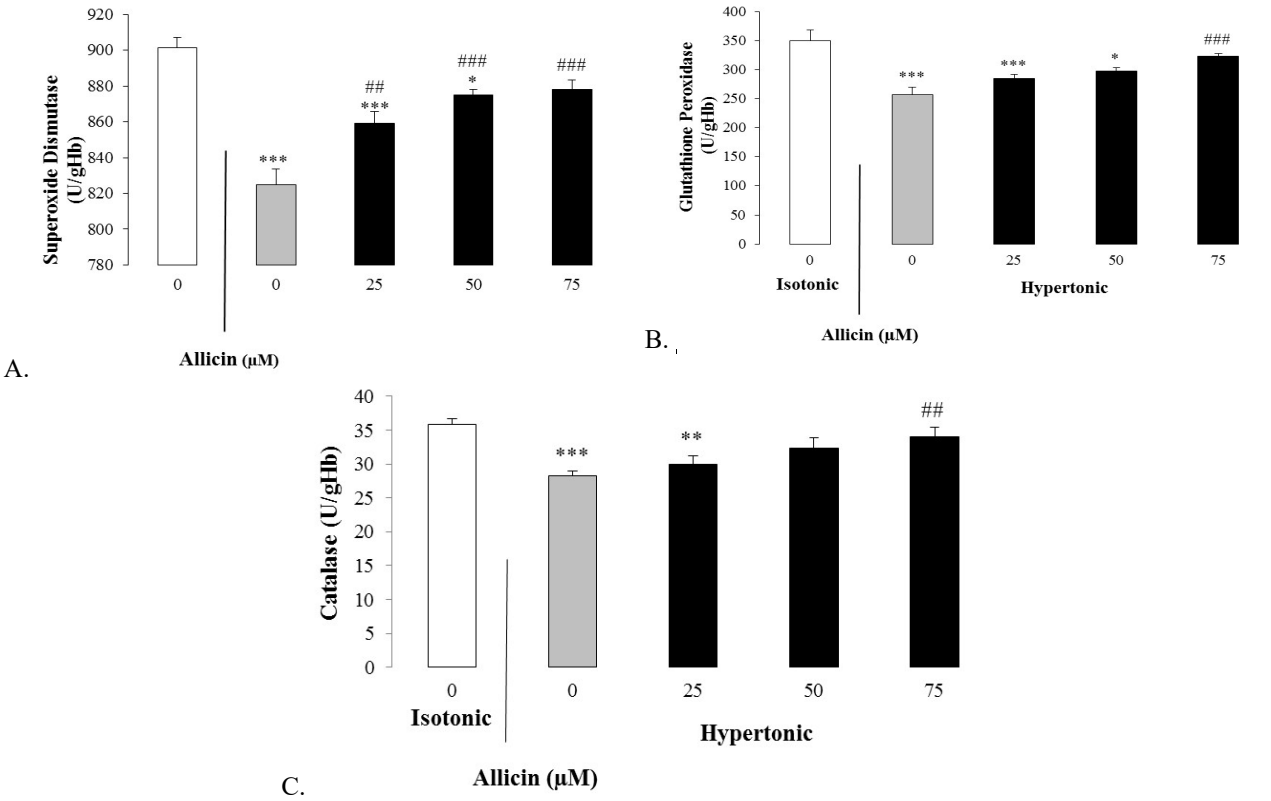

Fig. 1: Allicin's effect on (A) SOD, (B) GPx and (C) CAT activity of erythrocytes. Vertical bars represent arithmetic means ± SEM (n = 20) of enzymes activity. *, **, ***, ($p<0.05$, $p<0.01$, $p< 0.001$) showed statistical significance from isotonic control (white bar), ##, ### ($p<0.01$, $p< 0.001$) showed statistical significance from hypertonic control (grey bar) (ANOVA).

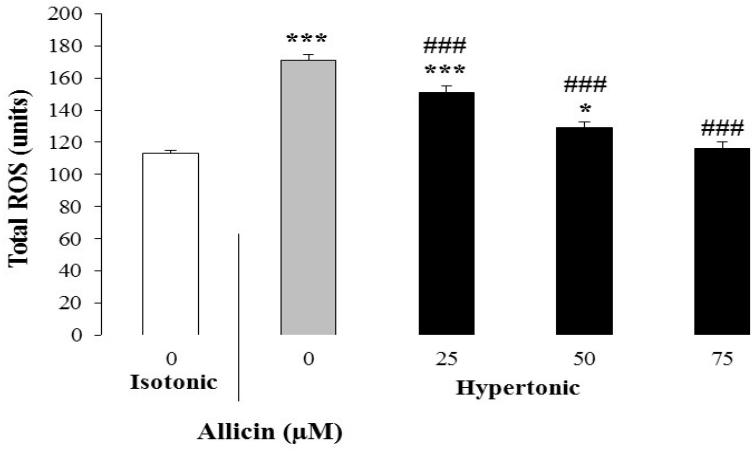

Fig. 2: Allicin's effect on ROS generation in erythrocytes. Vertical bars represent arithmetic means (units) ± SEM (n = 20) of ROS level. *, *** ($p<0.05$, $p< 0.001$) indicate statistical significance from isotonic control (white bar), ### ($p<0.001$) showed statistical significance from hypertonic control (grey bar)(ANOVA).

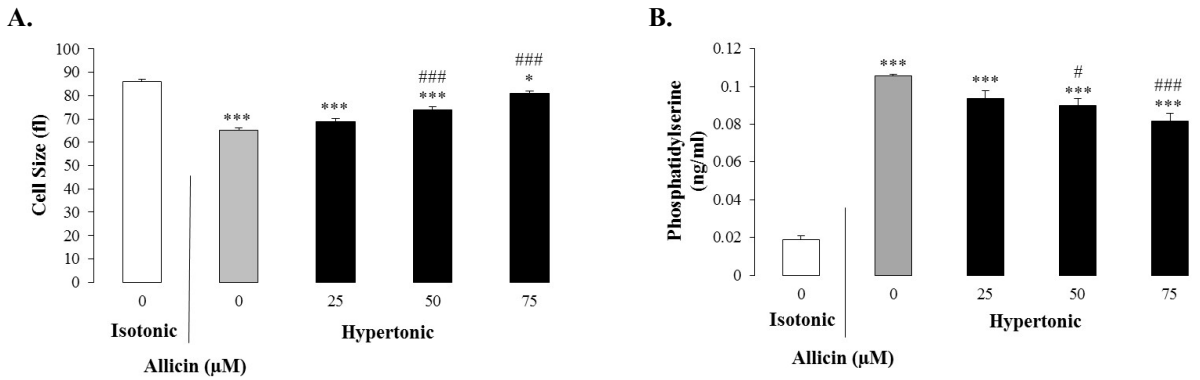

Fig. 3: Effects of allicin on erythrocyte's (A) cell size and (B) phosphatidylserine exposure (eryptotic markers). Vertical bars represent the arithmetic means ± SEM (n = 20) of change in erythrocyte's cell volume. *, *** ($p<0.05$, $p< 0.001$) represent statistical significance from isotonic control (white bar), #, ### ($p<0.05$, $p< 0.001$) represent statistical significance from hypertonic control (grey bar) (ANOVA).

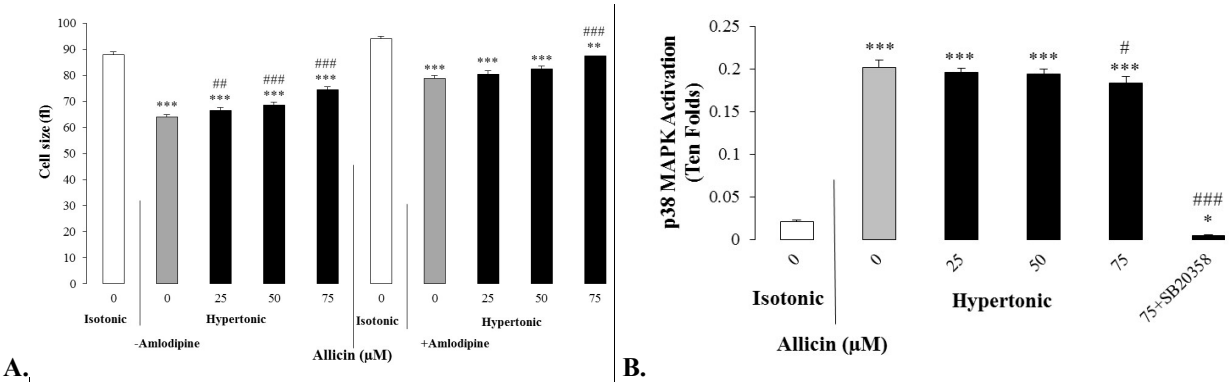

Fig. 4: Confirmation of underlying mechanisms involved in eryptosis (A) eryptotic role of Ca^{2+} (B) activation of p38MAPK (SB20358 is p38MAPK inhibitor) in the presence of allicin. Arithmetic means ± SEM (n = 20) of change in cell size. *, **, *** ($p<0.05$, $p<0.01$, $p< 0.001$) showed statistical significance from isotonic control (white bar), #, ##, ### ($p<0.05$, $p<0.01$, $p< 0.001$) showed statistical significance from hypertonic control (grey bar)(ANOVA).

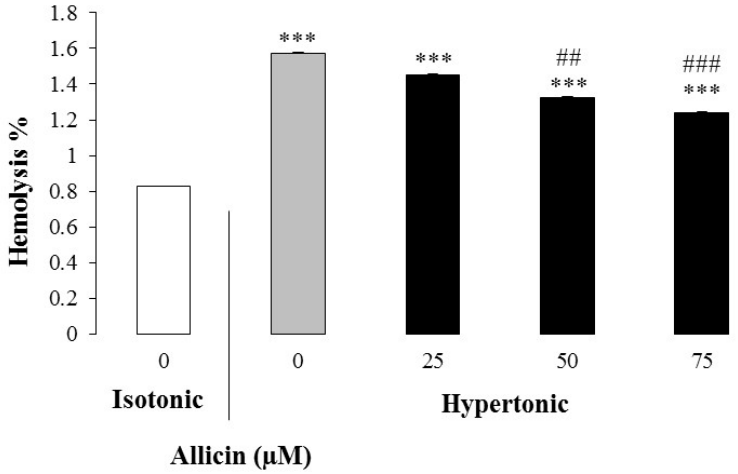

Fig. 5: Effect of hyperosmotic shock and allicin treatment on hemolysis % (cytotoxicity measurement). Vertical bars represent the % (n=20) of hemolysis. *** ($p<0.001$) indicate statistical significance from isotonic control (white bar), ##, ### ($p<0.01$, $p<0.001$) indicate statistical significance from hypertonic control (ANOVA).

The experimental results confirmed that hyperosmolarity enhances oxidative stress and reduces the activity of all antioxidants, while allicin treatment helps to enhance their activity and reduce ROS production. Reduction in ROS level due to Allicin is also exhibited by (Zhang *et al*., 2025[a]; Wang *et al.,* 2025; Zhang *et al.,* 2025[b]). Previous research has confirmed that antioxidant activity decreases with increased oxidative stress (Lucero *et al.*, 2015).

Erythrocyte's plasma membrane shrinkage and phosphatidylserine exposure are the main markers of eryptosis (Aslam *et al.*, 2024), while increased cytosolic Ca^{2+} and p38 MAPK activation plays vital role in the stimulation of eryptosis (Kurmangaliyeva *et al.,* 2025; Tkachenko *et al.,* 2025). Hyperosmolarity and oxidative stress contribute to the stimulation of Ca^{2+}channels, including p38 MAPK activation (Alghareeb and Alfhili, 2023). Studies showed that high oxidative stress and P-p38 MAPK stimulate Ca^{2+} channels located in erythrocytes' plasma membrane, thus regulating Ca^{2+} entry inside the cell (Li *et al.*, 2021). Increased level of cytosolic Ca^{2+} stimulates Ca^{2+}-dependent plasma membrane scrambling (phosphatidylserine exposure), including stimulation of Ca^{2+}-sensitive K^+ channels. Activation of the potassium channel leads to K^+ efflux and hyperpolarization of the plasma membrane, thereby promoting KCl release and H_2O efflux from the cell, causing cell shrinkage (Pretorius *et al.*, 2014). Previous studies confirmed allicin's ability to inhibit p38MAPK activation and its involvement in the reduction in oxidative stress in nucleated cells (Ba *et al.*, 2019; Nadeem *et al.*, 2021). Allicin treated erythrocytes showed reduced PS exposure, but the effect was partial, although p38MAPK activation and oxidative stress contributes in its externalization, but additional pathways (caspase dependent, ceramide signaling, PAF dependent stimulation of sphingomyelinases) are also involved in its externalization (Mandal *et al.,* 2005; Lang *et al.,* 2015; Tkachenko *et al.,* 2025). Previous research data and results of this study confirmed that hyperosmolarity stimulates p38MPAK and Ca^{2+} channels, thus stimulating the apoptotic mechanism, while allicin-treated erythrocytes showed better erythrocyte survival.

High oxidative stress induced by hyperosmolarity may cause structural protein oxidation (membrane and cytoskeletal proteins), potentially leading to hemolysis (Deng *et al.*, 2015; Wang *et al.*, 2021). In a recent study, (Ravindra *et al*., 2023) explained the antioxidant nature of allicin along with its scavenging ability for OH, including its ability to prevent lipid peroxidation (Ravindra *et al.*, 2023). Another study showed that hyperglycemia leads to ROS generation and ultimately results in myocardial infarction; allicin inhibits insulin resistance along with ROS generation (Nadeem *et al.*, 2021). According to results, allicin reduces the hemolysis rate, which may be due to its antioxidant nature, as ROS are the basic triggerer of plasma membrane oxidation and lead to hemolysis.

This study's efficacy lies in its thorough mechanistic approach, which integrates p38 MAPK signalling, apoptotic endpoints, oxidative stress markers and Ca^{2+} modulation within a single experimental framework. This study offers a comprehensive understanding of hyperosmolarity-induced eryptosis and its pharmacological control, in contrast to earlier research. Future *in-vivo* research and clinical studies are made possible by these findings to examine allicin or allicin-derived compounds as supplementary treatment agents for hyperosmolarity-associated anaemia, especially in metabolic illnesses like diabetes and chronic kidney disease.

This study covers a largely underexplored gap between hyperosmolarity and diabetes associated anemia by studying and modulating the p38 MAPK pathway by using least cytotoxic plant based compound, allicin as novel therapeutic approach to manage anemia. Investigating the anti-erythropoietic role of allicin will provide a generally safe, novel and nutraceutical-derived therapeutic intervention to treat hyperosmolarity-triggered anemia, along with its downstream microvascular, renal and cardiovascular complications.

CONCLUSION

This study suggests that allicin, a plant-derived compound with low cytotoxicity, may control elevated eryptosis during hyperosmotic stress by inhibiting p38 MAPK signaling and could be a candidate for treating hyperosmolarity-induced anemia in diabetic patients. Certain pathological conditions, like inflammatory, renal and metabolic, including diabetes, develop hyperosmolarity, which has been a major contributor to eryptosis, which, if neglected, leads to anemia. Anemia further complicates the situation by inducing tissue hypoxia, endothelial dysfunction and thrombosis, as well as leading to other complications, including cardiac and renal disorders and increased risk of foot ulcers and amputations. The present study confirmed that allicin attenuates/reduce hyperosmolarity-induced oxidative damage, ROS generation, p38 MAPK activation, Ca^{2+} dependent erythrocyte shrinkage, PS exposure and stabilizes erythrocyte membrane integrity. Results confirmed that allicin showed anti-eryptotic and cytoprotective effects and gave foundation for future clinical research targeting hyperosmolarity triggered anemia.

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Authors' contribution

Kashif Jilani and Maham Abdul Bari Khan: Designed the study; Maham Abdul Bari Khan: Investigated, analyzed, wrote, edited the data and organized the final draft; Kashif Jilani, Zahid Mushtaq and Hq Nawaz: Reviewed the data. All authors gave their final approval for this draft.

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Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethical approval

The study was conducted after the approval by the Institutional Bioethics Committee (IBC) and the Directorate of Graduate Studies (DGS) (approval no: 492/ Dated: 01-02-2024), University of Agriculture, Faisalabad, Pakistan.

Conflict of interest

There is no financial interest or personal relationship that could influence the work reported in this paper.

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