Clin. Lab. 2024;70:XXX-XXX
©Copyright

ORIGINAL ARTICLE

In Vitro Protective Effects of Total Extract and Fractions of Fenugreek (*Trigonella Foenum-Graecum* L.) on Red Blood Cells

Iman Morshedi

Department of Pharmacy, Tehran Branch, Islamic Azad University, Tehran, Iran

SUMMARY

Background: **Erythrocytes are susceptible to oxidative stress throughout their lifespan. While compounds like vitamin C can help mitigate oxidative stress, the exploration of natural herbal products continues to be a compelling area of research. To examine the impact of subfractions derived from acidified chloroform fractions of fenugreek (*Trigonella foenum-graecum* L.) on red blood cells in the presence of H_2O_2 as an oxidant, we assessed the factors associated with erythrocyte aging and oxidative stress.**

Methods: **The maceration technique was employed for extracting fenugreek seeds. Through chromatography, a total of 12 subfractions were isolated from the acidified chloroform extract of fenugreek seeds. Following an initial assessment, four subfractions exhibiting lower erythrocyte toxicity were chosen for further investigation. The objective was to evaluate their impact on erythrocyte aging by measuring the levels of phosphatidylserine (PS), sialic acid, CD47 on the erythrocyte surface, as well as oxidative stress biomarkers. The obtained results were presented as mean ± standard deviation (SD), and data analysis was performed by using ANOVA.**

Results: **The results of this study revealed, that among the 12 subfractions derived from the acidified chloroform fraction of fenugreek, four subfractions demonstrated protective effects against H_2O_2-induced hemolysis and oxidative stress. Furthermore, flow cytometry analysis indicated that treatment with three of these subfractions led to elevated levels of CD47 and reduced levels of phosphatidylserine on the surface of erythrocytes.**

Conclusions: **The results suggest that the subfractions of fenugreek extract which likely contain a higher concentration of flavonoids and a lower content of saponins could be responsible for the observed protection against erythrocyte aging processes. It appears that fenugreek seeds have the ability to safeguard human erythrocytes from oxidative damage by reducing oxidative stress, preserving the activity of antioxidative enzymes, and maintaining the integrity of erythrocyte structure.**

(Clin. Lab. 2024;70:xx-xx. DOI: 10.7754/Clin.Lab.2024.240421)

Correspondence:
Iman Morshedi
Department of Pharmacy
Tehran Branch
Islamic Azad University
Tehran
Iran
Email: imanmorshedi1@gmail.com

Manuscript accepted June 9, 2024

KEYWORDS

Trigonella foenum-graecum L., red blood cell, antioxidant activity, erythrocyte aging

INTRODUCTION

Erythrocytes are the most abundant visible components in the blood system. They usually have a lifespan of 120 days [1]. A reduced number of erythrocytes can cause anemia and lack of oxygen [2]. Since erythrocytes do not contain nuclei and mitochondria, oxidative stress usually causes membrane lipid peroxidation. Moreover, erythrocyte membranes are rich in unsaturated fats, and

hemoglobin contains more iron molecules [3]. Both are powerful catalysts of free radical reactions. Erythrocytes possess effective mechanisms to break down reactive oxygen species, thanks to the presence of superoxide dismutase. This enzyme converts O_2 into H_2O_2, which is then further broken down by catalase and glutathione peroxidase [4]. Therefore, erythrocytes are sensitive to oxidative damage from reactive oxygen species (ROS). Eliminating excess free radicals could reduce membrane lipid peroxidation and protect erythrocytes from oxidative injury [5]. Currently, there are limited known drugs that can protect erythrocytes from oxidative damage. However, chemical compounds extracted from plants, particularly medicinal plants used in Iran, have shown a promising potential [6,7]. Elevated oxidative stress induces alterations in membrane lipids and proteins, which may contribute to the onset of metabolic disorders. Oxidative stress is a state of biochemical imbalance that occurs as a result of an overabundance of free radicals and reactive oxygen species. This condition leads to increased oxidative damage to biomolecules, and the body's natural defense mechanisms, such as antioxidative systems, are unable to counteract it effectively [8]. Plants hold a universally acknowledged status as an essential component of the Earth's natural heritage. A staggering 80% of the population depends on plants for their primary healthcare needs. The diverse array of medicinal plants is acknowledged for their ability to provide natural antioxidants, which effectively safeguard against oxidative stress. As a result, these plants play a significant role in the prevention of various diseases through chemoprevention. The medicinal properties of plants can largely be attributed to the presence of flavonoids, coumarins, phenolic acids, as well as antioxidant micronutrients such as copper, zinc, and manganese [9]. Various types of medicinal plants are acknowledged as a valuable source of natural antioxidants, which offer protection against oxidative stress, and thereby play a crucial role in the prevention of diseases. Oxidative stress, resulting from the presence of free radicals, like reactive oxygen species (ROS), is now recognized as a fundamental mechanism underlying degenerative diseases such as diabetes, infections, autoimmune disorders, and aging. Scientific evidence suggests that ROS can be effectively scavenged through chemoprevention, utilizing antioxidant compounds found in both food and medicinal plants [10]. The effects of antioxidant compounds derived from herbal resources are diverse, offering multifaceted benefits. By consuming these compounds appropriately, we can effectively combat a range of metabolic imbalances [11]. Fenugreek (*Trigonella foenum-graecum* Linn.), belonging to the Fabaceae family, has demonstrated antioxidant activity in various animal models used for experimentation [12]. In this case, the primary outcome was the demonstration of protective effects against H_2O_2-induced hemolysis and oxidative stress by four subfractions derived from the acidified chloroform fraction of fenugreek. So, the objective of this study was to explore the protective effects of the methanol extract derived from *Trigonella foenum-graecum* on human red blood cells, using erythrocytes as a model system. The focus was to investigate the mechanisms and pathophysiology of oxidative damage and to evaluate the potential benefits of the mentioned extract in mitigating such damage.

MATERIALS AND METHODS

Plant collection, authentication, and extraction

The plant *Trigonella foenum-graecum* L. was gathered approximately 25 km from Kerman city, Iran, in September 2022. Subsequently, it underwent authentication by a plant taxonomist at the National Herbarium of the College of Natural and Botanical Sciences, Shahid Bahonar University.

The maceration method was utilized for the extraction process. Initially, 200 g of powdered leaves were weighed, using an analytical balance from Mettler Toledo, Switzerland. These leaves were then macerated in 1,000 mL of absolute methanol in an Erlenmeyer flask at a ratio of 1:5. After 3 days, the extract was collected and subjected to re-maceration. The collection of the extract was done every 3 days, resulting in a total maceration period of 9 days, with intermittent shaking on a rotary-shaker (Labnet, Germany). The pool of collected extracts underwent filtration, first through a sieve mesh and then through Whatman no. 1, using a filtration apparatus. The filtrate of extracts was concentrated in a rotary evaporator (Labnet, Germany) at a temperature of 40°C and a speed of 40 revolutions per minute (RPM) until all solvents were completely removed, resulting in the formation of solid extracts.

Crude extract solvent fractionation

The crude methanol extract underwent further solvent fractionation to increase polarity, utilizing n-hexane, chloroform, ethyl acetate, and aqueous solvents [13]. The method by Voukeng et al. (2017) was employed for solvent fractionation, with modifications made to the concentration of the extract residue between fractionation intervals [14]. The methanol extract (60 g) was accurately weighed on an analytical scale and completely dissolved in 100 mL of 90% methanol (10 mL water and 90 mL methanol) in a beaker. The fully dissolved 100 mL methanol extract was then combined with 100 mL n-hexane for solvent partitioning in a separatory funnel with a capacity of 250 mL. The mixture in the separatory funnel was allowed to settle until a clear and separated layer formed between the two solvents. Once the clear layer formed, the methanol part was carefully transferred to a beaker and the n-hexane partition was moved to another container. This process was repeated three times, and the n-hexane partition was collected altogether for future concentration. The process involved in the fractionation of the methanol extract was carried out meticulously. After evaporating the metha-

nol solvent in a rotary evaporator, water was added to create a 100 mL aqueous solution. This solution was then mixed with chloroform in a separatory funnel, allowing for the separation of the two layers. The chloroform portion was collected first, followed by the aqueous portion, and this process was repeated three times with the chloroform portion being pooled for concentration later. The remaining aqueous portion was concentrated to remove any residual chloroform. Subsequently, the concentrated aqueous portion was mixed with ethyl acetate in the separatory funnel, leading to the formation of distinct layers. This step was also repeated three times, with the aqueous and ethyl acetate fractions being collected separately in different containers [15].

DPPH radical scavenging activity

The DPPH assay is a method used to measure the antioxidant activity of a substance by evaluating its ability to scavenge free radicals. In this assay, the purple-colored stable free radical DPPH is reduced to yellow-colored diphenyl picryl hydrazine, which can be measured spectrophotometrically at 517 nm. To conduct the assay, a 1 mL solution of DPPH in methanol (0.1 mM) is mixed with a 3 mL sample solution in water at various concentrations. After a 30-minute incubation period, the absorbance of the mixture is measured at 517 nm. A decrease in absorbance of the DPPH solution indicates an increase in DPPH radical-scavenging activity. The IC50 value, which represents the concentration at which 50% scavenging is achieved, is determined by plotting the percentage of inhibition against the concentration of the sample. DPPH radical-scavenging activity was calculated according to the following equation: % Inhibition = $(A_0 - A_1)/A_0 \times 100$, where A_0 was the absorbance of the control (without extract) and A_1 was the absorbance in the presence of the extract [16].

TLC (thin layer chromatography)

Initially, various solvent systems were prepared. After necessary evaluation of the chloroform-ethyl acetate-methanol-formic acid (3:2:1:0.25) solvent system, it was used for washing the system. For this, 0.8 grams of ACC extract was dried for 42 hours at 45°C and loaded into a column packed with silica gel. The solvent exit rate from the column was adjusted to 1 mL/minute, and finally, methanol was used to elute all substances. The extracted components were collected in 0.1 mL microtubes. They were then placed for 48 hours, and a concentration of 1,000 ppm was spotted on TLC plates. UV radiation at wavelengths of 254 and 365 nm was used to examine the spots, and the fractions were identified. The obtained fractions were concentrated at 45°C [17].

Red blood cell lysis test

A concentration of 200 ppm of each of the saponin fractions was prepared in normal saline and ethanol. To 11 test tubes, 0.5 mL of blood was added and washed three times. To 10 tubes, 200 µL of saponin fraction and 1 mL of RPMI culture medium containing 1% penicillin/streptomycin were added. For the negative control group, 200 µL of normal saline and 1 mL of culture medium were added. The tubes were placed in an incubator at a temperature of 37°C and 5% CO_2. After 24 hours, for the positive control group, 200 µL of $2H_2O$ and 200 and 500 mM were added, and after 2 hours, all tubes were centrifuged, and the absorbance of the supernatant at a wavelength of 540 nm was read by the Eliza reader device. This process was repeated three times for each fraction [18].

The selected saponin fractions from the previous stage were prepared at concentrations of 50, 100, 200, 500, and 1,000 ppm with normal saline. Similar to the previous stage, in each tube, 5.0 mL of blood, 1 mL of RPMI culture medium containing 1% penicillin/streptomycin, and 200 µL of each specified concentration of saponin fractions were added. For the negative control group, 200 µL of physiological serum were added, and the tubes were placed in an incubator at a temperature of 37°C and 5% CO_2. After 42 and 84 hours, 200 µL of $2H_2O$ at 500 and 750 micromolar were added to the positive control group. After 2 hours, all tubes were centrifuged, and the absorption of the supernatant at a wavelength of 540 nanometers was read by an ELISA reader. This process was repeated three times for each fraction. To investigate the effects of H_2O_2 as a lysing agent, a similar procedure was followed. Except for the negative control, 0.02 µL of H_2O_2 with a concentration of 500 µM was added to all tubes. The resulting supernatant after centrifugation was considered for cell lysis assessment and was used for oxidative stress and aging tests [19].

Identification of saponin

The fractions obtained by TLC method in the saponin solvent system, including ammonia-ethanol-butanol (5/7-5/2-5/10), was carried out. Then, a 15% sulfuric acid solution was sprayed on the TLC paper as a reagent, and the plate was heated to a temperature of 120°C. The presence of saponins was confirmed when purple spots appeared on the surface of the plate [20].

Malondialdehyde level (MDA) measurement

The MDA method was utilized to assess the level of lipid peroxidation (LPO). A commercial kit from ZellBio GmbH, Germany, was employed for the analysis, following the manufacturer's instructions. This kit offers a simple, reproducible, and standardized approach to evaluate lipid peroxidation in biological samples. To conduct the analysis, all reagents were brought to room temperature, and samples were well-shaken for homogenization. Subsequently, 50 µL of standards/samples were added to the respective test tubes labeled with their names. Next, 50 µL of R4 and 1 mL of the ready chromogenic solution were added to the mixture, which was then incubated in a boiling water bath (90 - 100°C) for 1 hour. After cooling down, the samples were centrifuged at 3,000 - 4,000 rpm, and 200 µL of the pink-colored supernatant was pipetted into a microplate. The ab-

sorbance was measured by using a microplate reader/ ELISA reader at 535 nm. The MDA level in unknown samples was determined based on a standard curve generated from the absorbance of standard points [21].

Ferric reducing antioxidant power (FRAP) assay

The total antioxidant potential of a sample was determined by using the ferric reducing ability of FRAP assay as a measure of antioxidant power. The assay was based on the reducing power of a compound (antioxidant). A potential antioxidant will reduce the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}); the latter forms a blue complex (Fe^{2+}/TPTZ), which increases the absorption at 593 nm. Briefly, the FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), a solution of 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$ at 10:1:1 (*v*/*v*/*v*). The reagent (3.400 µL) and sample solutions (100 µL) were added to each well and mixed thoroughly. The absorbance was taken at 593 nm after 30 minutes [22].

Flow cytometry

The flow cytometry method was used to determine the surface phosphatidylserine and CD47 levels on red blood cells. To dilute the red blood cells, 5.0 mL of red blood cells were brought to a volume of 2 mL with physiological serum. To an assay tube, 57.4 mL of distilled water and 250 µL of Annexin V-FITC buffer were added. In the flow cytometry tubes, 50 µL of diluted red blood cells and 500 µL of diluted Annexin V-FITC buffer were added, followed by 3 µL of Annexin V-FITC, and incubated in darkness at room temperature for 15 minutes. The tubes were then directly injected into the flow cytometer (Partec PAS), and the labeled cells were counted as Annexin positive and analyzed, using flow cytometry software. To determine CD47, 50 µL of washed red blood cells were added to 5 µL of anti-CD47 antibody. The tube was then incubated for 10 minutes at 4°C, followed by the addition of 1 milliliter of phosphate saline buffer. All tubes were directly injected into the flow cytometer (Partec PAS), and the CD47-positive labeled cells were counted. The data were then analyzed, using flow cytometry software [23].

Statistical analysis

The standard calibration curve plotting, graph plotting, and data analysis for the research were conducted by using Microsoft Office Excel and SPSS (version 16). The data was presented as mean ± standard deviation. To compare experimental values, GraphPad Prism software (version 6) was used for statistical analysis, showing significant differences between groups using one-way ANOVA, with a p-value < 0.05 considered statistically significant.

RESULTS

The results of the DPPH test demonstrated that the chloroform fraction of the acid has effectively inhibited DPPH free radicals to a greater extent compared to other fractions, exhibiting stronger antioxidant effects (Figure 1). Based on the chromatography test, 12 subfractions were separated and identified (Table 1). The study of the effects of subfractions obtained on cell hemolysis compared to negative and positive control groups showed that subfractions 4, 10, 11, and 12 had the lowest level of hemolysis (Figure 2). The lytic effect of H_2O_2 in the presence of specific plant subfractions showed that subfractions 4, 10, 11, and 21 were able to significantly reduce the level of hemolysis (p-value ≤ 0.05) (Figure 3).

Flow cytometry findings for CD47 showed that after 24-hours contact in the presence of subfractions, only subfraction 10 had a significant difference with the control group (p-value ≤ 0.001). This means that subfraction 10, unlike other subfractions, was not able to compensate for the decrease due to H_2O_2 for CD47 (Figure 4).

The flow cytometry findings for phosphatidylserine indicated that the level of apoptosis in red blood cells after 24 hours of contact with subfractions showed a significant difference only for subfraction 10 (p-value ≤ 0.001). This means that unlike other subfractions, subfraction 10 was unable to reduce the level of apoptosis to the extent observed in the negative control group (Figure 5).

In this study, the amount of sialic acid changes in the positive control groups, subfraction 4 (100 ppm), subfraction 10 (50 ppm), subfraction 11 (50 ppm), and subfraction 21 (200 ppm), in comparison with the control group, was significant ($p \leq 0.001$), and all subfractions were able to compensate for the decrease in sialic acid level compared to the positive control group. The level of FRAP decreased significantly after receiving H_2O_2, compared with the control group ($p < 0.05$). These changes increased significantly after treatment with subfraction 10 with a concentration of 50 ppm, compared to the control group ($p < 0.05$). There was a significant difference in LPO changes compared to the positive and negative controls ($p \leq 0.001$), and all subfractions decreased the LPO level compared to the positive control group (Table 2).

DISCUSSION

Erythrocytes possess distinctive characteristics that enable them to effectively break down reactive oxygen species. This is accomplished through the action of various enzymes and molecules, including superoxide dismutase, catalase, glutathione/glutathione peroxidase, methemoglobin reductase, and membrane-bound α-tocopherol [24]. However, under conditions of oxidative stress, the erythrocyte membrane becomes susceptible

Table 1. Characteristics of the subfractions separated from the column chromatography of the ACC fraction of fenugreek seeds.

Subfraction	Value (mg)	Solvent system
1	0.012	Methanol-formic acid-chloroform-ethyl acetate (2:3:0/25:1)
2	0.015	Methanol-formic acid-chloroform-ethyl acetate (2:3:0/25:1)
3	0.017	Methanol-formic acid-chloroform-ethyl acetate (2:3:0/25:1)
4	0.0428	Methanol-formic acid-chloroform-ethyl acetate (2:3:0/25:1)
5	0.0107	Methanol-formic acid-chloroform-ethyl acetate (2:3:0/25:1)
6	0.0373	Methanol-formic acid-chloroform-ethyl acetate (2:3:0/25:1)
7	0.0229	Methanol-formic acid-chloroform-ethyl acetate (2:3:0/25:1)
8	0.0116	Water-ethyl acetate-formic acid-acetic acid (1:1:1/1:10:2)
9	0.017	Water-ethyl acetate-formic acid-acetic acid (1:1:1/1:10:2)
10	0.0401	Water-ethyl acetate-formic acid-acetic acid (1:1:1/1:10:2)
11	0.0342	Water-ethyl acetate-formic acid-acetic acid (1:1:1/1:10:2)
12	0.0318	Water-ethyl acetate-formic acid-acetic acid (1:1:1/1:10:2)

Table 2. Comparison of oxidative stress changes in four subfractions and control groups.

Variables	Negative control	Positive control	Sub fraction 4 (100 ppm)	Sub fraction 10 (50 ppm)	Sub fraction 11 (50 ppm)	Sub fraction 12 (200 ppm)
Sialic acid mg/g (hemolysate protein)	716.5 ± 8.26	494 ± 10.69 ***	568.9 ± 8.38 ***†††	555.2 ± 5.29 ***†††	561.9 ± 16.49 ***†††	542.4 ± 8.08 ***†††
Lipid peroxidation µmol/mg (hemolysate protein)	1.837 ± 0.09	4.447 ± 0.06 ***	3.017 ± 0.077 ***†††	2.647 ± 0.13 ***†††	2.822 ± 0.20 ***†††	2.445 ± 0.08 ***†††
mmol Fe (III)/gr (hemolysate protein)	6.307 ± 0.28	5.563 ± 0.24 *	5.01 ± 0.38 ***	6.373 ± 0.52 †	5.797 ± 0.14	5.962 ± 0.14

The results are shown as means ± SD. * p < 0.05 and *** p < 000.1 compared to the negative control group and † p < 0.05 and ††† p < 000.1 compared to the positive control group.

to lipid peroxidation, leading to the formation of malondialdehyde (MDA). MDA serves as a biomarker for studying lipid oxidation under different conditions. When exposed to hydrogen peroxide and tert-butyl hydroperoxide, erythrocytes experience increased concentrations of MDA and decreased levels of intracellular reduced glutathione (GSH). These alterations in MDA and GSH concentrations in stressed erythrocytes indicate an elevated pro-oxidant/antioxidant ratio compared to normal erythrocytes [9]. The objective of this novel study conducted in Iran was to explore the impact of isolating subfractions of fenugreek seeds (*Trigonella foenum-graecum*) through chromatography and assessing their protective properties against cell lysis induced by H_2O_2 (an oxidizing agent). The ACC fraction was mainly composed of saponins and flavonoids from fenugreek seeds. Therefore, considering the surfactant effect of saponins, the goal was to select subfractions of the plant that have the least amount of saponins. The TLC results of the subfractions did not show positive results for separating saponins, so further biological tests were conducted on all subfractions for continuation of the work. There are different methods to investigate the antioxidant effects of substances [25]. One of the methods is to examine their effects on blood separated from the study samples. For initial hemolysis screening, RBCs were exposed to 200 ppm of the subfractions for 24 and 84 hours. Four subfractions, including 4, 10, 11, and 12, which exhibited the least hemolysis in their presence, were used. It is likely that these subfractions have lower amounts of saponins or a lower ratio of saponins to plant flavonoids. When RBCs were exposed to concentrations of 10, 50, 100, 200, 500, and 1,000 ppm of subfractions 4, 10, 11, and 12 for

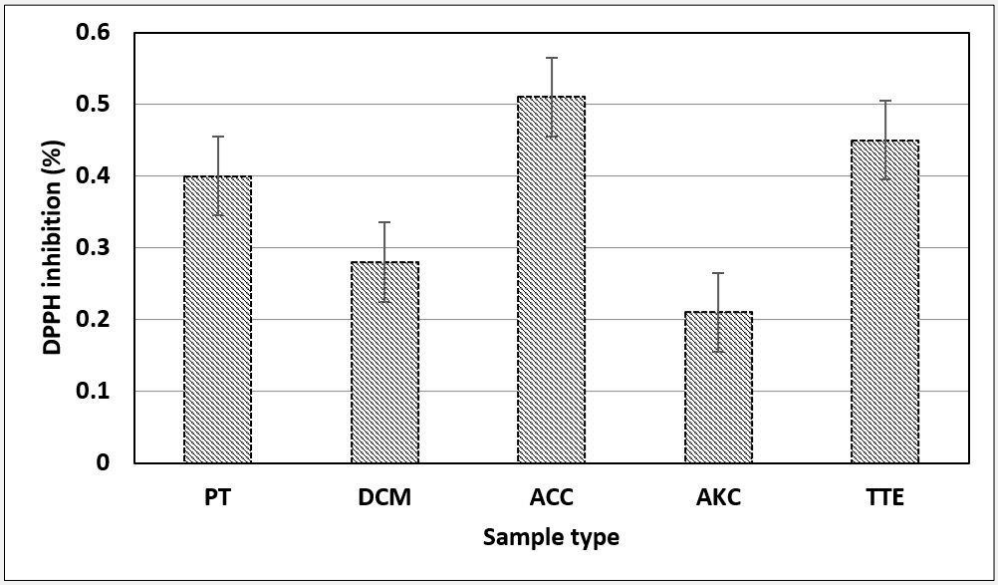

Figure 1. Comparison of the DPPH radical inhibitory level of different fractions of *Trigonella foenum-graecum* L.

PT - petroleum ether fraction, DCM - dichloromethane fraction, AKC - alkaline chloroform fraction, ACC - acidic chloroform fraction, TTE - the total extract.

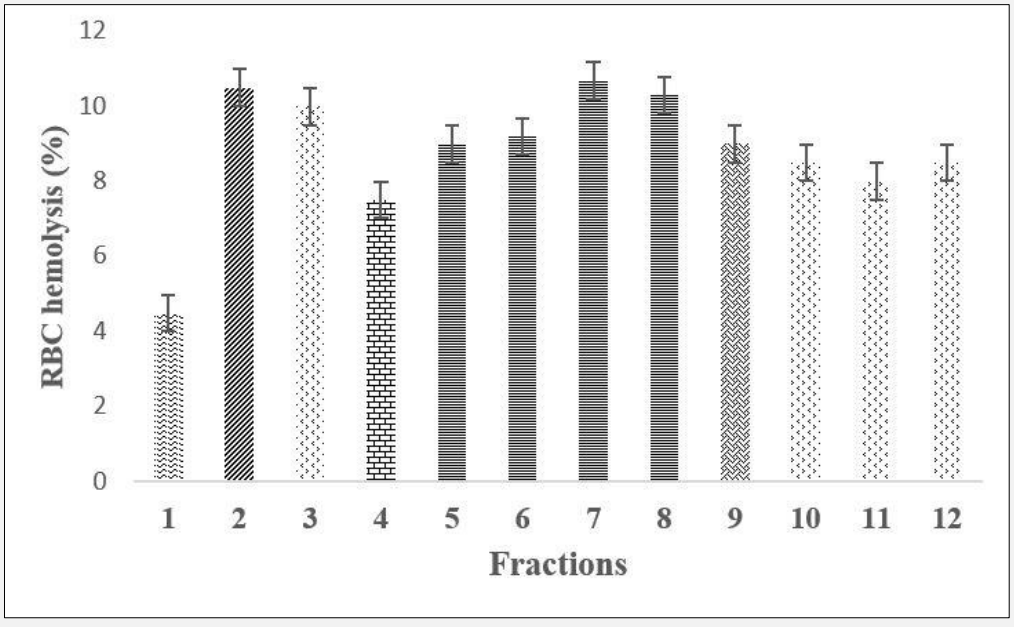

Figure 2. Evaluation of the level of hemolysis of ACC subfractions prepared from fenugreek seeds at a concentration of 200 ppm, compared to the control group (* p-value ≤ 0.01, ** p-value ≤ 0.01, *** p-value ≤ 0.005).

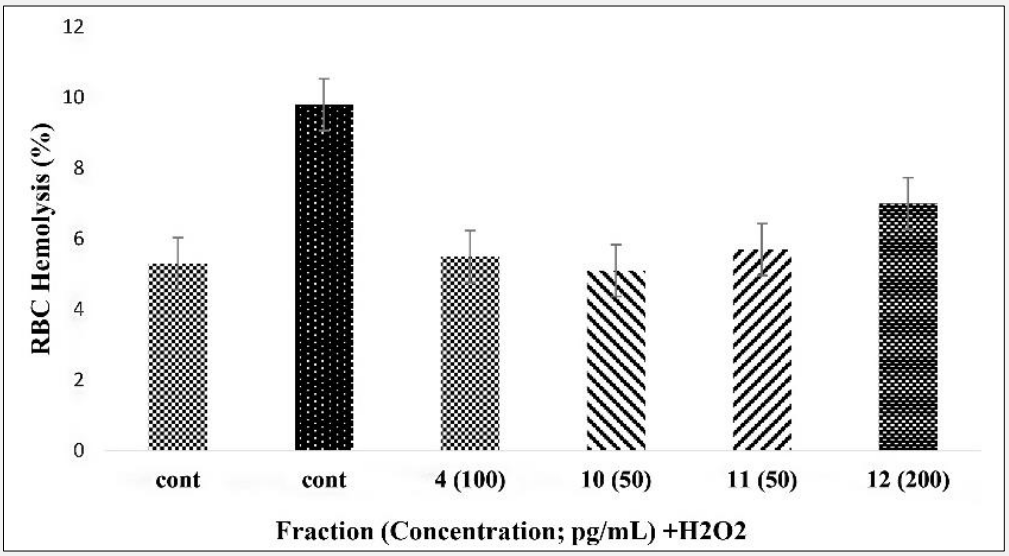

Figure 3. Protective effect of specific concentrations of subfractions 10, 4, 11, and 12 in the presence of H_2O_2, compared to the control group (p-value ≤ 0.05).

24 hours, all subfractions caused hemolysis at a concentration of 1,000 ppm. However, different subfractions showed varying degrees of inhibition of hemolysis in the presence of H_2O_2 at different concentrations, demonstrating stronger effects compared to the positive control in some cases. In general, subfractions 4, 10, 11, and 12 demonstrated the highest protective effect against hemolysis. This suggests that these subfractions may have stronger effects in inhibiting hemolysis compared to the positive control in certain instances. Further analysis and research may be needed to understand the mechanisms behind these varying effects and their potential implications. One of the initial experiments conducted by Stocks and Dormandy used RBCs to investigate the ability of H_2O_2 to induce hemolysis and lipid peroxidation [26]. Another investigation focused on studying the protective effects of dietary fenugreek seeds on the erythrocytes of streptozotocin-induced diabetic rats. The study provides valuable insights into the protective effects of dietary fenugreek seeds and onions on erythrocytes in streptozotocin-induced diabetic rats. This investigation specifically aimed to evaluate how these dietary components could mitigate such adverse effects. This study underscores the potential of dietary fenugreek seeds and onions as protective agents for erythrocytes in diabetic conditions. Their combined effects not only enhance the antioxidant capacity but also improve the structural integrity of red blood cells, making them a promising dietary intervention for managing diabetes-related complications. The synergistic effect observed with the combination of fenugreek and onion suggests that incorporating these foods into the diet could provide enhanced benefits for individuals suffering from diabetes and related hematological issues [27]. Moreover, findings from a separate study revealed that fenugreek seed extract possesses antioxidants, safeguarding cellular structures against oxidative harm. Fenugreek was observed to lower hemolysis rates in H_2O_2-treated red blood cells, suggesting its protective role in preserving the integrity of the red blood cell membrane. Fenugreek mitigates oxidative stress-induced damage to the delicate RBC membrane caused by H_2O_2 [28]. In another study, the polyphenol content and antioxidant capacity of aqueous extracts from eight medicinal plants were investigated, along with their protective effects against oxidative stress in red blood cells and preadipocytes. The data indicate that decoction slightly enhanced polyphenol extraction and antioxidant capacity compared to infusion extraction (as assessed by the DPPH test). However, infusions showed a superior protective effect against oxidative stress-induced red blood cell hemolysis [29]. High levels of unsaturated fatty acids in RBC membranes and the high concentration of hemoglobin make these cells susceptible to oxidative stress, particularly when exposed to oxidizing agents such as H_2O_2. The released hemoglobin from RBCs reacts with H_2O_2 and becomes oxidized, leading to hemolysis and the release of iron ions. In other words, H_2O_2 can act as an initiator for the formation of free radicals in the presence of iron, which subsequently convert unsaturated fatty acids into free radicals through peroxidation [30]. In the study conducted by Kaviarasan

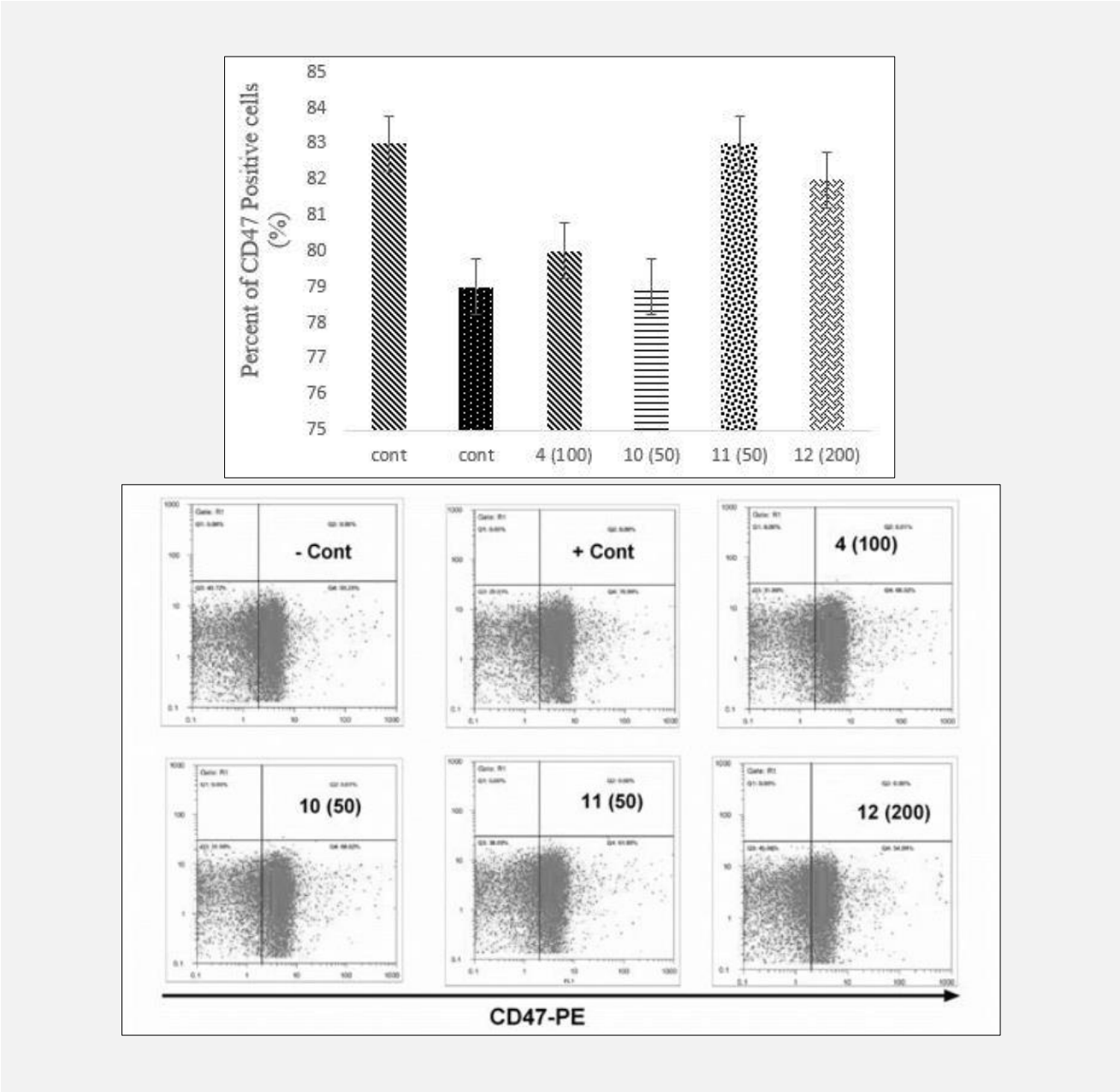

Figure 4. Flow cytometric analysis of CD47 in RBC compared to the control group (p-value ≤ 0.001).

et al., the methanolic extract of the Shambhala plant seed was used. The number of polyphenolic compounds was measured by using the Singleton and Rossi method, and the extent of hemolysis and lipid peroxidation induced by H_2O_2 was investigated in the presence of this plant extract in both diabetic and healthy individuals. According to this study, the fraction rich in phenolic compounds from the Shambhala seed contains significant amounts of flavonoids [31]. In a study conducted by Tedesco et al., the protective effects of red wine against oxidative stress induced by H_2O_2 were observed in RBCs [32]. Red wine is also rich in polyphenolic compounds such as catechin, gallic acid, quercetin, and resveratrol. It appears that these polyphenolic compounds in red wine are responsible for its protective effects against oxidative damage [33]. Contact of RBCs with agents that induce peroxidation of unsaturated fatty acids in their membranes can lead to increased membrane rigidity and fragility, as well as a decrease in the cell's ability to change shape. This may be due to a reduction in certain intracellular compounds, such as ATP, and an increase in TBARS (thiobarbituric acid reactive substances). A decrease in ATP levels can result in a diminished ability of the cell to maintain ion ho-

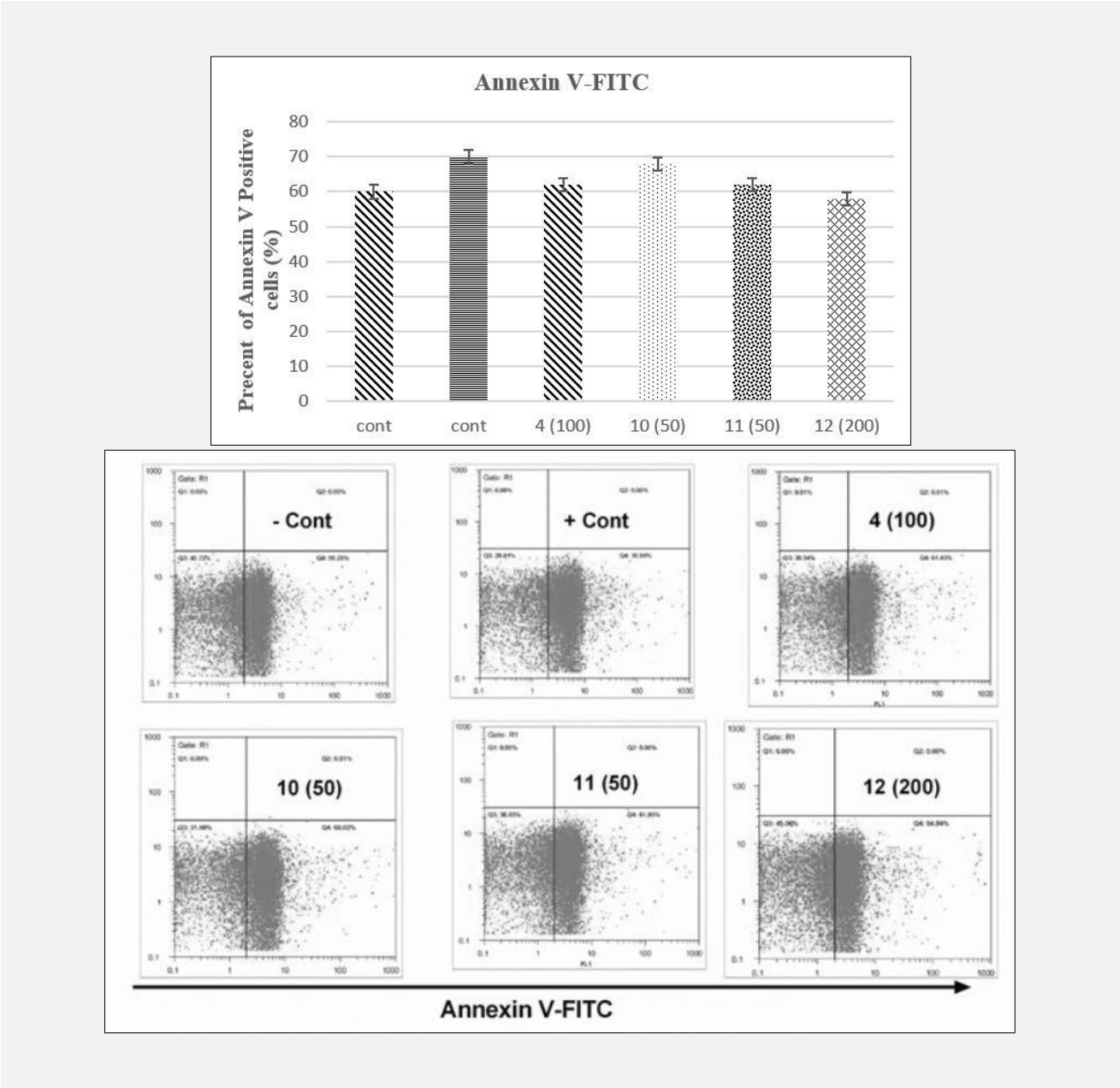

Figure 5. Flow cytometric analysis of phosphatidyl serin in RBC compared to the control group (p-value ≤ 0.001).

meostasis and preserve its proper shape. Additionally, an increase in TBARS formation is indicative of oxidative damage to the cell, suggesting that it has been subjected to increased production of free radicals and oxidative stress [19]. In another study, it has been shown that garlic extract can reduce lipid peroxidation induced by tert-butyl hydroperoxide in RBCs, thereby inhibiting the reduction in the ability of RBCs to change shape [34]. In a separate study, an extract derived from potato peels, containing polyphenolic compounds like caffeic acid and gallic acid, was utilized. The results demonstrated significant protective effects of the extract in preserving membrane integrity and reducing lipid peroxidation in red blood cells (RBCs). This suggests that the polyphenolic compounds present in the potato peel extract play a beneficial role in safeguarding RBCs from oxidative damage and maintaining their overall health [35]. The results obtained from investigating the effects of selected subfractions on oxidative stress tests, CD47 factors, and phosphatidylserine in the present study demonstrated that all subfractions exhibited protective and antioxidant effects compared to the positive control group (H_2O_2) within a specific concentration range, following the hormetic phenomenon. An increase

in the mobility of CD47 leads to its clustering on the erythrocyte membrane. This clustering phenomenon has been observed specifically in apoptotic erythrocytes. However, the exact effects of CD47 clustering on the inhibition or facilitation of clearance mechanisms are still not well understood. This suggests that the subfractions have potential benefits in mitigating oxidative stress and promoting antioxidant effects, which could have implications for various health conditions. Ongoing investigations are currently exploring these different possibilities to gain a deeper understanding of the role of CD47 clustering in the associated mechanisms. In this research, secondary outcomes include the flow cytometry analysis results showing elevated levels of CD47 and reduced levels of phosphatidylserine on the surface of erythrocytes after treatment with three of the subfractions. These results are important, because they indicate a potential positive impact of the treatment with three of the subfractions on the surface of erythrocytes. The flow cytometry analysis showing elevated levels of CD47 and reduced levels of phosphatidylserine suggests that the treatment may have beneficial effects on the cells. This information could be significant in understanding the mechanisms of action and potential therapeutic applications of the subfractions in question.

CONCLUSION

The results of this study indicate that different subfractions derived from the ACC fraction of the Shambhala seed can have protective effects. Based on studies conducted on subfractions of the plant and the investigation of the effects of plant compounds on RBC aging factors, such as CD47, it seems that further research should focus on these subfractions, especially subfractions 10, 11, and 12, for identification and isolation of the active compound under study. These findings provide scientific backing for the traditional use of *Trigonella foenum-graecum* in mitigating oxidative stress, which is implicated in metabolic disorders and their related complications. By examining both the total extract and fractions of fenugreek, the study provides a comprehensive analysis of the potential benefits of this plant on RBCs. Additionally, the use of different fractions allows for a more detailed understanding of the specific components responsible for any observed effects. Overall, the study's specific focus and methodology contribute to its strength in exploring the protective effects of fenugreek on red blood cells. Exploring medicinal plants as a potential economically sustainable source of potent and natural antioxidants offers promising prospects in combating complications associated with oxidative stress. Furthermore, such research outcomes have the potential to enhance our understanding of the ethnopharmacological properties of medicinal plants.

Declaration of Interest:
The author declares that he has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References:

1. Corrons JLV, Casafont LB, Frasnedo EF. Concise review: how do red blood cells born, live, and die? Ann Hematol 2021; 100(10):2425-33. (PMID: 34342697)

2. Kuhn V, Diederich L, Keller TCS, et al. Red blood cell function and dysfunction: redox regulation, nitric oxide metabolism, anemia. Antioxid Redox Signal 2017;26(13):718-42. (PMID: 27889956)

3. Naparlo K, Bartosz G, Stefaniuk I, Cieniek B, Soszynski M, Sadowska-Bartosz I. Interaction of catechins with Human erythrocytes. Molecules 2020;25(6):1456. (PMID: 32213847)

4. Mohanty JG, Nagababu E, Rifkind JM. Red blood cell oxidative stress impairs oxygen delivery and induces red blood cell aging. Front Physiol 2014;5:84. (PMID: 24616707)

5. An F, Wang S, Yuan D, Gong Y, Wang S. Attenuation of oxidative stress of erythrocytes by plant-derived flavonoids, orientin and luteolin. Evid Based Complement Alternat Med 2016;2016: 3401269. (PMID: 26966458)

6. Bahmani M, Shirzad H, Majlesi M, Shahinfard N, Rafieian-Kopaei M. A review study on analgesic applications of Iranian medicinal plants. Asian Pac J Trop Med 2014;7S1:S43-53. (PMID: 25312163)

7. Jaberian H, Piri K, Nazari J. Phytochemical composition and *in vitro* antimicrobial and antioxidant activities of some medicinal plants. Food Chem 2013;136(1):237-44. (PMID: 23017418)

8. Sadiq IZ. Free radicals and oxidative stress: signaling mechanisms, redox basis for human diseases, and cell cycle regulation. Curr Mol Med 2023;23(1):13-35. (PMID: 34951363)

9. Luqman S, Kaushik S, Srivastava S, et al. Protective effect of medicinal plant extracts on biomarkers of oxidative stress in erythrocytes. Pharm Biol 2009;47(6):483-90. https://www.tandfonline.com/doi/full/10.1080/13880200902832900

10. Akbari B, Baghaei-Yazdi N, Bahmaie M, Mahdavi Abhari F. The role of plant-derived natural antioxidants in reduction of oxidative stress. BioFactors 2022;48(3):611-33. (PMID: 35229925)

11. Hano C, Tungmunnithum D. Plant polyphenols, more than just simple natural antioxidants: Oxidative stress, aging and age-related diseases. Medicines (Basel) 2020;7(5):26. (PMID: 32397520)

12. Kumar P, Bhandari U. Protective effect of *Trigonella foenum-graecum* Linn. on monosodium glutamate-induced dyslipidemia and oxidative stress in rats. Indian J Pharmacol 2013;45(2):136-40. (PMID: 23716888)

13. Safdar MN, Kausar T, Nadeem M. Comparison of ultrasound and maceration techniques for the extraction of polyphenols from the mango peel. J Food Process Pres 2016;41(4):e13028. https://ifst.onlinelibrary.wiley.com/doi/10.1111/jfpp.13028

14. Voukeng IK, Nganou BK, Sandjo LP, et al. Antibacterial activities of the methanol extract, fractions and compounds from *Elaeophorbia drupifera* (Thonn.) Stapf. (Euphorbiaceae). BMC Complement Altern Med 2017;17(1):28. (PMID: 28061888)

15. Kebede B, Shibeshi W. *In vitro* antibacterial and antifungal activities of extracts and fractions of leaves of *Ricinus communis* Linn against selected pathogens. Vet Med Sci 2022;8(4):1802-15. (PMID: 35182460)

16. Sannigrahi S, Mazuder UK, Pal DK, Parida S, Jain S. Antioxidant potential of crude extract and different fractions of *Enhydra fluctuans* Lour. Iran J Pharm Res 2010;9(1):75-82. (PMID: 24363710)

17. Alenazy R. Antimicrobial activities and biofilm inhibition properties of *Trigonella foenumgraecum* methanol extracts against multidrug-resistant *Staphylococcus aureus* and *Escherichia coli*. Life (Basel) 2023;13(3):703. (PMID: 36983858)

18. Chatterjee S, Premachandran S, Bagewadikar RS, Poduval TB. The use of ELISA to monitor amplified hemolysis by the combined action of osmotic stress and radiation: potential applications. Radiat Res 2005;163(3):351-5. (PMID: 15733043)

19. Remigante A, Spinelli S, Straface E, et al. Antioxidant activity of quercetin in a H2O2-induced oxidative stress model in red blood cells: functional role of band 3 protein. Int J Mol Sci 2022; 23(19):10991. (PMID: 36232293)

20. Zheng Y-F, Qi L-W, Zhou J-L, Li P. Structural characterization and identification of oleanane-type triterpene saponins in *Glycyrrhiza uralensis* Fischer by rapid-resolution liquid chromatography coupled with time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 2010;24(22):3261-70. (PMID: 20973000)

21. Amrollahi N, Enshaei Z, Kavousi F. Salivary malondialdehyde level as a lipid peroxidation marker in early childhood caries. Iran J Pediatr 2021;31(4):e113824. https://brieflands.com/articles/ijp-113824

22. Ou B, Huang D, Hampsch-Woodill M, Flanagan JA, Deemer EK. Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: a comparative study. J Agric Food Chem 2002;50(11):3122-8. (PMID: 12009973)

23. Burger P, Hilarius-Stokman P, de Korte D, van den Berg TK, van Bruggen R. CD47 functions as a molecular switch for erythrocyte phagocytosis. Blood 2012;119(23):5512-21. (PMID: 22427202)

24. Winterbourn CC, Kettle AJ, Hampton MB. Reactive oxygen species and neutrophil function. Annu Rev Biochem 2016;85: 765-92. (PMID: 27050287)

25. Zeb A. Concept, mechanism, and applications of phenolic antioxidants in foods. J Food Biochem 2020;44(9): e13394. (PMID: 32691460)

26. Stocks J, Dormandy T. The autoxidation of human red cell lipids induced by hydrogen peroxide. Br J Haematol 1971;20(1):95-111. (PMID: 5540044)

27. Pradeep SR, Srinivasan K. Haemato-protective influence of dietary fenugreek (*Trigonella foenum-graecum* L.) seeds is potentiated by onion (*Allium cepa* L.) in streptozotocin-induced diabetic rats. Biomed Pharmacother 2018; 98:372-81. (PMID: 29275179)

28. Dinakaran V, Sridharan S, Periyasamy L, Rani SS. In vitro antioxidant effects of Trigonella foenum graecum (L.) Fenugreek seed extract on sheep red blood cells. J Med Plants Res 2012; 6(38):5119-27. https://academicjournals.org/journal/JMPR/article-abstract/AFAAB7A16800

29. Checkouri E, Reignier F, Robert-Da Silva C, Meilhac O. Evaluation of polyphenol content and antioxidant capacity of aqueous extracts from eight medicinal plants from Reunion Island: Protection against Oxidative Stress in Red Blood Cells and Preadipocytes. Antioxidants (Basel) 2020;9(10):959. (PMID: 33036442)

30. Cheng Z, Li Y. What is responsible for the initiating chemistry of iron-mediated lipid peroxidation: an update. Chem Rev 2007; 107(3):748-66. (PMID: 17326688)

31. Kaviarasan S, Vijayalakshmi K, Anuradha CV. Polyphenol-rich extract of fenugreek seeds protect erythrocytes from oxidative damage. Plant Foods Hum Nutr 2004;59(4):143-7. (PMID: 15678722)

32. Tedesco I, Russo M, Russo P, et al. Antioxidant effect of red wine polyphenols on red blood cells. J Nutr Biochem 2000;11(2): 114-9. (PMID: 10715597)

33. Nemzer B, Kalita D, Yashin AY, Yashin YI. Chemical composition and polyphenolic compounds of red wines: their antioxidant activities and effects on human health-a review. Beverages 2022; 8(1):1. https://www.mdpi.com/2306-5710/8/1/1

34. Davidović-Plavšić B, Kukavica B, Škondrić S, Jimenez-Gallardo C, Žabić M. Wild garlic extract reduces lipid peroxidation in terbuthylazine-treated human erythrocytes. Biomarkers 2021;26(7): 617-24. (PMID: 34253103)

35. Sampaio SL, Petropoulos SA, Dias MI, et al. Phenolic composition and cell-based biological activities of ten coloured potato peels (*Solanum tuberosum* L.). Food Chem 2021;363:130360. (PMID: 34153675)