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Short communication

Reduction of oxidative stress of normal and G6PD enzyme-deficient human erythrocytes by *Centaurea ammocyanus* extract (*in vitro* study)

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ABSTRACT

Centaurea species have received considerable interest in biological researches due to their many important biological activities. As a continuation of our studies on *Centaurea* species, the current study aimed to assess *Centaurea ammocyanus* aerial parts hydromethanolic extract for its phytochemical constituents, determine its total phenolic content, investigate its hemolytic effect on human erythrocytes and anti-hemolytic activity in the protection of normal and G6PD enzyme-deficient human erythrocytes against oxidative damage. The results demonstrated that the total phenolic content of *C. ammocyanus* extract was 37.65± 0.6 mg gallic acid equivalent/g extract. The results revealed that hemolysis caused by *C. ammocyanus* extract was very low with a maximum value of 4.06% at the concentration 3000 µg/ml. The results also showed that the hemolysis induced by H_2O_2 is reduced in a concentration-dependent manner in the presence of the *C. ammocyanus* extract. The highest protection values were about 88.14% and 92.59% for normal and G6PD-deficient erythrocytes, respectively. Through this study, it can be concluded that *C. ammocyanus* aerial parts extract showed no obvious hemolytic effect, and could be considered safe for human erythrocytes. The extract had antioxidant activity and was able to protect normal and G6PD-deficient human erythrocytes against hemolytic damage induced by hydrogen peroxide.

1. INTRODUCTION

Erythrocytes are the major blood cells[1]. They are exclusive cells that carry oxygen to our body as well as participating detoxification of variety of xenobiotics[2]. This specialized function of transport oxygen (O_2) and carbon dioxide (CO_2), is known to be the major one of red blood cells, there are also many important functions such as delivery of nitric oxide[1].

Erythrocytes hemolysis is caused due to the disruption of their membrane resulting in the release of hemoglobin and other internal components into the surrounding fluid[3].

Hemolysis occurs in many pathological conditions such as mechanical disruption of erythrocytes, malaria/clostridium infection, autoimmunity against erythrocytes surface antigen,

sickle cell and thalassemia disease[4]. Furthermore, erythrocytes are susceptible to oxidative injury because their membranes contain high levels of polyunsaturated fatty acids[5]. Free radicals attack erythrocytes and lead to membrane changes including lipid peroxidation, changes in cell morphology, reduction in deformability. These changes cause lysis of erythrocytes[6].

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common enzymopathy, affecting approximately 400 million people worldwide. It is X-linked, genetic disorder, caused by mutations in the G6PD gene[7]. Males are much more likely than females to have G6PD deficiency which is most prevalent in Asia, Africa, the Middle East, and the Mediterranean[8]. G6PD-deficient patients are at risk of hemolytic anemia when exposed to oxidative stress, while newborn infants show prolonged jaundice, both cases may lead to death[9]. Most G6PD-deficient patients do not show any manifestations until following exposure to oxidative drugs, ingestion of Fava bean and some infections[10].

Generally, Phytochemicals constitute an important class of plant-derived compounds with beneficial health properties because of their anti-oxidant activities among other biological properties[11]. Some *Centaurea* species are used in folk medicine and this is due to the secondary compounds they contain[12]. The plant *Centaurea ammocyanus* Boiss. belongs to *Centaurea* genus (Asteraceae). It is annual herb[13], (5-30 cm)[14]. The central branch frequently reduced to a sessile head, the lateral prostrate. Scales of involucre glabrous. Flowerets pink[13]. Achenes 2-2.5 mm[14]. It spreads in sandy places[13].

As a continuation of our studies on *Centaurea* species, this study aimed to screen *C. ammocyanus* plant for its phytochemical constituents, evaluate its hemolytic effect on human erythrocytes, and anti-hemolytic activity in the protection of normal and G6PD enzyme-deficient human erythrocytes against oxidative hemolysis induced by H_2O_2. Based on our knowledge, this is the first report about the assessment of the hemolytic effect, and the anti-hemolytic activity of this plant.

2. METHODOLOGY

2.1. Collection of plant material:

The aerial parts of *C. ammocyanus* were collected from Syria. The plant material was authenticated based on the morphological characteristics mentioned in flora of Syria, Palestine and Sinai[13], as well as flora of Syria and Lebanon[14]. Then, it was dried and powdered for optimum extraction.

2.2. Extraction procedure:

Plant material (50 g) was extracted with 250 ml of aqueous methanol (80%) as a solvent, using ultrasound assisted extraction technique. The frequency was constant at 30 kHz for 25 min. The extract was filtered. The filtrate was evaporated using rotary evaporator[15]. Finally, the crude extract was dissolved in Phosphate buffer saline (PBS) to prepare the required concentrations for subsequent tests.

2.3. Phytochemical analysis:

A preliminary phytochemical analysis was performed for the detection of phenols (lead acetate test[16]), flavonoids (Shinoda test[17]), saponins (foam test[17]), tannins (gelatin test[18]), (ferric chloride test[19]), carbohydrates (Molisch test[20]).

2.4. Total phenolic content (TPC):

TPC was measured using the Folin-Ciocalteu's method[21]. Gallic acid was used as a standard. The total phenolic content was expressed as gallic acid equivalent (GAE) in milligrams per gram of dry extract.

2.5. Blood samples collection:

Erythrocytes were collected from venous blood of donors after their consent. They were collected from healthy donors for hemolysis test and from healthy donors and G6PD deficiency patients for anti-hemolytic assessment. Erythrocyte were separated by centrifugation and then suspended in sterile PBS to obtain the erythrocytes suspension (10 %) to be used later in subsequent tests.

2.6. Hemolysis test:

Hemolytic effect of the hydromethanolic extract of *C. ammocyanus* was evaluated by spectrophotometer method. A volume of 1 ml of the erythrocytes suspension was added to 1 ml of the

plant extract (100, 250, 500, 1000, 2000, 3000 µg/ml). The reaction mixtures were incubated (30 min, 37°C) and then centrifuged. The free hemoglobin found in the supernatant was used for measurement at 540 nm. Distilled water and PBS were used as positive and negative hemolytic controls, respectively. The percentage hemolysis was calculated as follows[22]:

$$\text{hemolysis\%} = [(\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{control-}}) / (\text{Absorbance}_{\text{control+}} - \text{Absorbance}_{\text{control-}})] * 100$$

control-: PBS; control+: distilled water.

2.7. Anti-hemolytic assessment:

The ability of *C. ammocyanus* hydromethanolic extract to prevent oxidative hemolysis was determined as a measure of *in vitro* antihemolytic effect. A volume of 0.5 ml of normal erythrocytes suspension was added to 1 ml of the plant extract (100-3000 µg/ml). Then 0.5 ml of hydrogen peroxide solution (prepared in PBS) of appropriate concentration was added. After incubation (37°C, 240 min) tubes were centrifuged. After that, the free hemoglobin found in the supernatant was used for measurement at 540 nm. In view of the encouraging results obtained in the test applied on normal erythrocytes, the study was followed up by investigating the activity of the plant in the protection of erythrocytes isolated from patients with G6PD deficiency against oxidative hemolysis. For this purpose, we selected only three concentrations (1000-2000-3000 µg/ml) of plant extract due to their ability to provide high protection ratios in the test applied on normal erythrocytes. To compare the results in both tests, two control samples were prepared, which were positive control sample (erythrocytes suspension, Ascorbic acid, and hydrogen peroxide solution), and negative one (erythrocytes suspension, PBS, and hydrogen peroxide solution). The percentage of protection (anti-hemolytic activity) was calculated as follows[23]:

Hemolysis%= (Absorbance of sample / Absorbance of control) x100
Protection %= 100 – [hemolysis]

The IC50 value, responsible for 50% inhibition of hemolysis induced by H_2O_2 was determined.

2.8. Statistical analysis:

The results were expressed as mean± standard deviation. The average values were calculated from ten replicates for normal erythrocytes and seven replicates for G6PD enzyme-deficient erythrocytes. Statistical Package for Social Science (SPSS) software was used for analyzing data. The level of significance was set at ($P<0.05$).

3. RESULTS

3.1. Phytochemical analysis:

Preliminary phytochemical screening results showed that *C. ammocyanus* aerial parts hydromethanolic extract contain phenolic compounds, tannins, flavonoids, carbohydrates and little amount of saponins (Table 1).

Table 1. Characteristics of included studies

Constituent		*C. ammocyanus*
Tannins	Ferric chloride	+
	Gelatin test	+
Carbohydrate		+
Flavanoids		+
Phenolic compounds		+
Saponins		•

(•) little presence, (+) presence

3.2. Total phenolic content (TPC):

The result demonstrated that *C. ammocyanus* extract contain good phenolic content with a value of 37.65± 0.6 mg gallic acid equivalent/g of dry extract (mg GAE/g).

3.3. Hemolytic effect:

The results showed that the plant extract was safe in the field of low concentrations (100-500) µg/ml, as it did not show any significant differences in comparison with the negative control. However, at the higher concentrations (1000-3000 µg/ml) , hemolysis values increased but did not exceed about 4% at the highest concentration (3000 µg/ml). Significant differences between extract and negative control are represented in (Table 2).

Table 2. The percentages of hemolytic effect by *C. ammocyanus* extract

C. ammocyanus (µg/ml)	concentration Hemolysis%
0.21±0.2	100
0.32±0.3	250
0.51±0.1	500
2.34*±1.4	1000
2.99*±1.2	2000
4.06*±0.9	3000

All values are represented as mean ± SD, (*) Significant differences at ($P<0.05$)

3.4. Anti-hemolytic activity:

The results showed that the human erythrocytes undergo hemolysis when exposed to H_2O_2 without plant extract (con -). The hemolysis induced by H_2O_2 is reduced in a concentration-dependent manner in the presence of the *C. ammocyanus* extract. In the test applied on normal erythrocytes, the extract showed clear protection in the field of concentrations between 1000 to 3000 ug/ml with the value of IC50 = 629.74 (Table 3). The plant extract showed relevant anti-hemolytic effect in comparison of Ascorbic acid as positive control (data not shown). However, the protection values were remarkably increased in the case of enzyme-deficient erythrocytes. The highest protection values were about 88.14% and 92.59% for normal and G6PD-deficient erythrocytes respectively. We used Ascorbic acid in this test as positive control because it is well known as antioxidant agent. The results showed the activity of ascorbic acid in the protection of G6PD-deficient human erythrocytes at the concentration of 1000 ug/ml and 2000 ug/ml, while its ability was clearly decreased at the highest concentration 3000 µg/ml. Significant differences between extract and positive control are represented in Table 4.

Table 3. The percentages of hemolysis and protection by *C. ammocyanus* extract against oxidative hemolysis

Normal human erythrocytes			
Concentration	***C. ammocyanus***		**IC50**
(µg /ml)	**Hemolysis%**	**Protection%**	**(µg /ml)**
100	83.56	14.44±1.7	629.74
250	79.26	20.74±3.4	
500	64.31	35.69±3.9	
1000	39.99	60.01±5.2	
2000	14.53	85.47±1.2	
3000	11.86	88.14±2	

All values are represented as mean ± SD

Table 4. Inhibition of lysis of G6PD enzyme-deficient human erythrocytes in presence of *C. ammocyanus* extract and ascorbic acid

G6PD enzyme-deficient human erythrocytes				
Concentration	***C. ammocyanus***		**Ascorbic acid**	
(µg /ml)	**Hemolysis%**	**Protection%**	**Hemolysis%**	**Protection%**
1000	10.42	89.58*±1.9	5.89	94.11±2.4
2000	9.96	90.04*±1.6	4.24	95.76±1.4
3000	7.41	92.59*±2.1	43.4	56.6±3.8

All values are represented as mean ± SD, (*) Significant differences at $P<0.05$

4. DISCUSSION

Phytochemicals are naturally present in the plant and make it useful for treating many diseases[23]. Phytochemical analysis revealed that *C. ammocyanus* aerial parts hydromethanolic extract contain carbohydrates, phenols, flavanoids, tannins, and saponins. The result of the total phenolic content determination test showed that the extract contained good level of phenolic compounds, which are known to have antioxidant properties.

Toxicity test for active molecules is an important step in the field of drug design, and hemolytic activity assessment represents a useful tool in this regard[24]. Due to the similarity of the red blood cells membrane content with most of animal cell membranes[1], hemolysis test has been widely used as a model for studying the interaction of drugs with cellular membranes[25]. It presents a direct indication of toxicity of injectable formulations[26], and is considered very sensitive for cytotoxic studies with wide range of phytochemicals[27]. In our study, hemolysis assay is used to evaluate the toxicity of *C. ammocyanus* aerial parts. Analyzing the results, the plant extract showed no obvious hemolytic effect. the supernatants did not show red coloration and all tested tubes had a red blood cell precipitate at all tested concentrations, therefore the extract did not cause hemolysis and could be considered safe for human erythrocytes. This may encourage the use of this plant, which has many important biological activities, in the pharmaceutical preparations.

Reactive oxygen species (ROS) including super oxides, hydroxyl radicals, hydrogen peroxide cause oxidative stress and damage cell Membrane[10]. One major role of antioxidants is protecting the cells from oxidative stress which results from an imbalance between the formation of ROS and the body antioxidant defense[28]. RBC has been used as a model to study the interaction between oxidants and antioxidants since their membranes contain high levels of polyunsaturated fatty acids, which are susceptible to peroxidation[29]. In our present study, the ability of *C. ammocyanus* aerial parts extract to protect human red blood cells from oxidative hemolysis was tested.

The treatment of red blood cells with H_2O_2 (toxicant) resulted in rupture of their membranes and thus caused visual hemolysis. When the normal erythrocytes were incubated with the plant extract in the presence of hydrogen peroxide, remarkable decrease in the hemolysis was observed in comparison with negative control. This result revealed that *C. ammocyanus* extract has potential inhibitory capacity against oxidative hemolysis.

A deficiency in G6PD enzyme was associated with accumulation of cellular ROS[30]. This enzyme catalyzes the first step in pentose phosphate pathway (PPP) that converts glucose-6-phosphate to 6-phosphogluconate with production of nicotinamide adenine dinucleotide phosphate (NADPH)[31]. The (PPP) pathway is the only source of NADPH in erythrocytes[32]. NADPH is necessary for generating of reduced glutathione (GSH) from its oxidized form, GSSG[8]. Glutathione peroxidase uses GSH to remove peroxides from red blood cells[10]. In the current study, when G6PD-deficient erythrocytes were incubated with the plant extract in the presence of hydrogen peroxide, the plant extract showed clear activity in reducing the oxidative damage. Despite the high protection values provided by the extract, these values did not exceed those provided by Ascorbic acid which was known for its antioxidant capacity. When G6PD-deficient

erythrocytes were incubated with ascorbic acid in the presence of hydrogen peroxide, marked reduction in hemolysis was noticed at the concentrations 1000-2000 µg/ml, while at the highest concentration 3000 µg/ml, the hemolysis increased. Our findings are in agreement with previous study which showed that ascorbic acid -when used with high concentrations- may interact with erythrocytes causing lipid peroxidation of membrane, oxidation of hemoglobin and thus hemolysis[33].

In general, the activity of the extract in the protection of erythrocyte membrane is due to its ability to act as antioxidant, possibly due to its containment of antioxidant compounds. Recently, phenolic compounds including flavonoids, phenolic acids and tannins have been well recognized as antioxidants[34].

5. CONCLUSION

Through this study it can be concluded that *C. ammocyanus* aerial parts extract showed no obvious hemolytic effect, and could be considered safe for human erythrocytes. The results also revealed that the extract had antioxidant activity and was able to protect normal and G6PD-deficient human erythrocytes against hemolytic damage induced by hydrogen peroxide. It could be suggested that *C. ammocyanus* aerial parts extract can be a supportive agent against the high consumption of endogenous body antioxidants in the case of oxidative injury. Further studies of this plant are wanted (in vivo), as it may provide a new therapeutic agent for favism disorder. Studies to verify its helpfulness are in progress.

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