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Phytochemical Analysis, Total Phenolic Content, Hemolytic and Anti-hemolytic Activities of *Centaurea iberica* (Asteraceae)

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ABSTRACT

Several plants contain chemical substances that have various biological activities, but they also probably cause harmful effects on cellular membranes. This work deals with the *Centaurea iberica* (Iberian starthistle) plant. The objective of this study is to identify the main chemical groups in the methanolic extract 80% of the plant, determine its total phenolic content, evaluate its toxic effect on human erythrocytes, and also assess its activity in the protection of normal and glucose-6-phosphate dehydrogenase (G6PD) enzyme-deficient human erythrocytes membranes against oxidative hemolysis. Phytochemical analysis revealed that *C. iberica* extract contains several bioactive compounds. The results also showed that the plant extract contained good level of total phenolic compounds. The extract exhibited a low hemolytic effect. It also showed excellent activity in the protection of normal and G6PD-deficient human erythrocytes against oxidative hemolysis.

KEYWORDS
Erythrocytes, G6PD, Iberian starthistle, methanolic extract, toxic effect, oxidative hemolysis

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1. Introduction

Since ancient time, plants are used as source of shelter and food for human (Kalita *et al.*, 2011). Plants contain different components which are used in traditional medicine, and several modern drugs have natural origin (Lohith *et al.*, 2013).

Although Syria is not a big country, it is rich in animal and plant diversity. This is due to its climatic and topographic diversity. More than 3500 species belonging to 131 plant families have been found in this country, hundreds of which might have medicinal importance (Alachkar *et al.*, 2011). *Centaurea* genus is in the family Asteraceae, comprising about 400 to 700 species (Kilic, 2013). Their purple, blue, yellow, or orange flower heads are color spots in many Mediterranean habitats and are appreciated photo subjects by many travelers (Hilpold, 2011). Members of the *Centaurea* genus are used as ornamentals such as *Centaurea cyanus* (Tomar, 2017) and *Centaurea cineraria*. Some *Centaurea* species are used in folk medicine and this is due to the secondary compounds they contain (Hilpold, 2011). The term "Novel Weapons Hypothesis" was proposed to describe the general effects of *Centaurea* species (Khammar and Djeddi., 2012). *Centaurea iberica* is an annual or biennial herb (20-80 cm), with pale pink, flowers (Davis, 1975). It contains many phytochemical compounds. Previously, flavones, fatty acids, steroids, volatile constituents, and sesquiterpene lactones have been reported from this plant (Khan *et al.*, 2011). It was known for its uses in folk medicine for the cure of peptic ulcer, malaria, stomach upset, common cold, abdominal pain, and herpes infections, and suggested against inflammatory situations such as asthma and abscesses (Khammar and Djeddi., 2012). On the other hand, Previous investigations on different extracts of this plant have shown cytotoxic activity (Dumlu and Gürkan., 2006).

The cytotoxic effect of many plants on human erythrocytes has previously been tested. The extract of *Allium strachey*i Baker prepared using butanol as a solvent, was reported to have a high cytotoxic effect (100%) on human erythrocytes, at the concentration of (500 µg/ml) (Mukherjee and Rajasekaran., 2010). In another study, the extract of *Bridellia Ferruginea* prepared using hexane as a solvent, was reported to have a hemolytic effect (92%) at the concentration (80 ng/ml) (Vinjamuri *et al.*, 2015).

This study investigated the phytochemical components in *C. iberica* extract, evaluated its total phenolic content, its hemolytic effect, and anti-hemolytic activity through the inhibition of induced oxidative hemolysis. This research forms part of our detailed study on some *Centaurea* species (doctoral thesis).

2. Materials and Methods

2.1. Plant Material Collection:

Arial parts of *C. iberica* were collected from the countryside of Aleppo city (Al Sfera). The plant material was authenticated and air-dried. Then, it was ground well to powder.

2.2. Preparation of the Hydro Alcoholic Extract:

50 g of plant powder was mixed with 250 ml of aqueous methanol 80% in a sealed container. Then the container was put in an ultrasonic waves bath (Hwashin Technology, Korea) for 25 min (30 kHz, 25^{O}C). The extract obtained was filtered using the Whatman-No.-1 filter paper in the sintered glass Büchner funnel under low pressure. The crude extract was obtained by evaporating the solvent in a rotary evaporator (Heidolph Instruments, Germany) under reduced pressure at 40 ^{O}C and kept in a desiccator. The extract was weighed to calculate the percentage of the extraction yield (Sangeetha and Vidhya, 2016).

2.3. Qualitative Analysis of *C. iberica* Extract:

Phytochemical screening of the aerial parts of *C. iberica* was conducted. Few amount of *C. iberica* extract was dissolved in distilled water and then filtered. Phytochemical constituents screening was performed using the filtrate.

2.3.1. Saponins

Few amount of filtrate (10 ml) was shaken vigorously. The presence of saponins was indicative if foam appeared and persisted for 10 minutes (Mojab *et al.*, 2003).

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2.3.2. Phenolic compounds

Few amount (3 ml) of lead acetate solution (10%) was added to 5 ml of filtrate. The presence of phenolic compounds was indicative if bulky white precipitate developed (Sangeetha and Vidhya, 2016).

2.3.3. Tannins

Few drops of ferric chloride solution (5%) were added to the filtrate. The presence of tannins was indicative if green color appeared (Shwetha *et al.*, 2016).

For Gelatin test, 1% gelatin solution containing 10% sodium chloride was prepared, then few drops of this solution were added to the filtrate, the presence of tannins was indicative if white precipitate developed (Pandey and Tripathi, 2014).

2.3.4. Flavonoids

Little amount of the filtrate (5 ml) was treated with few magnesium turnings and drops of concentrated hydrochloride acid HCl. The presence of flavonoids was indicative if pink color developed (Mojab *et al.*, 2003).

2.3.5. Carbohydrates

Few drops of methanolic alpha-naphthol (1%) were added to the filtrate (5 ml), and shaked well. Then concentrated Sulfuric Acid was added to the tube, and the presence of carbohydrates was indicative if a violet ring appeared at the junction (Wani *et al.*, 2012).

2.4. Determination of Total Phenolic Content (TPC):

TPC in the hydromethanolic extract of *C. iberica* was determined by the Folin–Ciocalteu method as described by (Alhafez *et al.*, 2014): To 1ml of the extract, 4.8 ml of distilled water was added, followed by the addition of 4 ml of sodium carbonate Na_2CO_3 (2%) and 200 µl of Folin–Ciocalteu reagent. The mixture was incubated for 60 minutes at 25°C. Then, the absorbance of the mixture was measured at 760 nm using a spectrophotometer. A blank sample was prepared using distilled water instead of *C. iberica* extract. The TPC of the extract was determined in four replicates. Gallic acid solutions (50-450 mg/L) were used as standard. TPC in the dry extract was quantified from the standard calibration curve of gallic acid solutions, and expressed as milligrams of gallic acid equivalents per gram of dry extract.

2.5. Cytotoxic effect on human erythrocytes (hemolytic effect assessment) (Sahu *et al.*, 2014):

1 ml of different concentrations of *C. iberica* extract (100-250-500-1000-2000-3000 µg/ml) was transferred by micropipette to test tubes containing a fixed volume (1 ml) of red blood cell suspension (10%). Test tubes were incubated for thirty minutes in a water bath at 37°C. Then, tubes were centrifuged by laboratory centrifuge for ten minutes at 2500 rpm (Heraeus Megafuge, Germany), and the absorbance was determined spectrophotometrically at 540 nm (Shimadzu, Japan). The hemolytic effect of the extract was evaluated by comparison with the positive control (distilled water) and negative control (PBS). The hemolysis percentage was calculated using the equation:

Percentage hemolysis = $[(Abs_s-Abs_p) / (Abs_d-Abs_p)] *100$

Abs_s- Absorbance of sample.

Abs_p- Absorbance of PBS.

Abs_d- Absorbance of distilled water.

Note: the erythrocytes suspension and the concentrations of the *C. iberica* extract were prepared in phosphate buffer saline (PBS) [KH_2PO_4 (0.24 g), NaCl (8 g), Na_2HPO_4 (1.44 g), KCl (0.2 g) dissolved in 1 liter of distilled water, pH=7.4] (Kuhlmann, 2006).

2.6. Inhibition of H_2O_2-induced hemolysis (anti-hemolytic activity) (Kavitha and Lena, 2014):

The human erythrocyte hemolysis was performed with hydrogen peroxide (H_2O_2) as a free radical initiator. 0.5ml of erythrocytes suspension (10%) was added to 1ml of *C. iberica* extract of different concentrations (100-250-500-1000-2000-3000 µg/ml), and the tubes were incubated for five minutes at room temperature. To each tube, 0.5 ml of H_2O_2 (in PBS pH 7.4) was added (the concentration of H_2O_2 was prepared to obtain about 90% hemolysis after 4 h incubation). Likewise, the erythrocytes were treated with 1 ml of PBS and 0.5 ml H_2O_2 and without plant extract to obtain complete hemolysis (blank). Test tubes were incubated at 37°C for four hours. Then, centrifuged at 2500 rpm for 10 min. Finally, the absorbance (abs) of the supernatants was measured spectrophotometrically at 540 nm. The inhibitory effect of the extract was compared with positive control (standard antioxidant Ascorbic acid) and negative control (PBS). The 50% inhibitory concentration (IC50) values were calculated as the antioxidant concentration required for the inhibition of 50% of hemolysis.

The percentage of protection was determined by the equation:

Protection%= 100 – [(Abs of test sample / Abs of control) *100]

The same procedure was done to investigate the activity of the extract in the protection of G6PD enzyme-deficient human erythrocytes against oxidative hemolysis. In this test, three concentrations of plant extract were chosen (1000-2000-3000) µg/ml. These concentrations were selected because they showed the highest activities in the previous test on the normal erythrocytes.

3. Results

3.1. Yield Extraction and Phytochemical Analysis:

After extraction, *C. iberica* extract resulted in 15.46% yield. The extract was characterized by a yellowish-green color and a pleasant grassy smell.

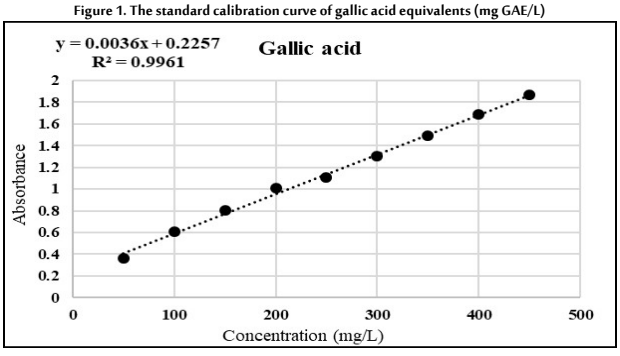
Phytochemical screening revealed that *C. iberica* extract contains several bioactive compounds which display several pharmacological and biological activities. It revealed the presence of saponins, phenolic compounds, flavonoids, tannins and carbohydrates.

3.2. Total Phenolic Content (TPC):

TPC in *C. iberica* aerial parts extract was determined by the Folin-Ciocalteu method. Total phenolics determination was based on the absorbance value of the extract solution (0.01 g/ml) that reacted with the Folin-Ciocalteu reagent, followed by a reference to the standard calibration curve of gallic acid (figure 1), according to the following equation: $y = 0.0036x + 0.2257$, $R^2 = 0.9961$

The Result implied that *C. iberica* aerial parts hydromethanolic extract contains good phenolic content with a value of 40.09± 0.52 mg gallic acid equivalent/g of extract (mg GAE/g).

Figure 1. The standard calibration curve of gallic acid equivalents (mg GAE/L)



3.3. Cytotoxic Effect on Human Erythrocytes:

C. iberica hydromethanolic extract exhibited a low hemolytic effect on human erythrocytes in comparison to negative control, so it is probably considered safe for human erythrocytes at low concentrations. The absorbance value was increased gradually with an increase in plant extract concentration (Table 1). Significant differences between extract and negative control were noticed at the range of concentrations (500-3000 µg/ml).

Table 1. The percentages of hemolytic activity by *C. iberica* extract

concentration (µg/ml)	*C. iberica* Hemolysis%
100	1.32 ± 1.6
250	2.09 ± 2.3
500	4.83* ± 2.9
1000	6.71* ± 2.0
2000	9.06* ± 0.9
3000	10.05* ± 1.1

All values are represented as mean ± SD
*Correlation is significant at the 0.05 level

3.4. Inhibition of H_2O_2 Induced Hemolysis (Anti-hemolytic Activity):

When red blood cells were incubated with the standard antioxidant (Ascorbic acid) and H_2O_2, a marked reduction in hemolysis was noticed at the range of concentrations of (250-3000 µg/ml), while at the highest concentration (3000 µg/ml) hemolysis was found to be increased as well as the protection was decreased (table 2, figure 2). The IC50 value of Ascorbic acid was 230.9 µg/ml (figure 3).

Table 2. The percentages of hemolysis and protection by Ascorbic acid and *C. iberica* against oxidative hemolysis

Concentration (µg /ml)	Ascorbic acid		IC50 (µg /ml)
	Hemolysis%	Protection%	
100	79.7475	20.25±1.5	230.9
250	43.9875	56.01±3.9	
500	23.8775	76.12±3.6	
1000	9.9575	90.04±1.7	
2000	5.3775	94.62±2.1	
3000	21.65	78.35±2.6	
Concentration (µg /ml)	*C. iberica*		IC50 (µg /ml)
	Hemolysis%	Protection%	
100	85.81	14.19*±2.77	623.41
250	79.738	20.26*±1.72	
500	61.262	38.74*±2.03	
1000	41.52	58.48*±2.02	
2000	15.452	84.55*±3.43	
3000	10.27	89.73*±2.24	

Protection values are represented as mean ± SD
*Correlation is significant at the 0.05 level

Figure 2. Anti-hemolytic activity of Ascorbic acid against oxidative hemolysis

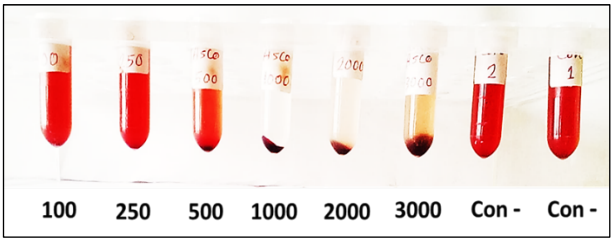
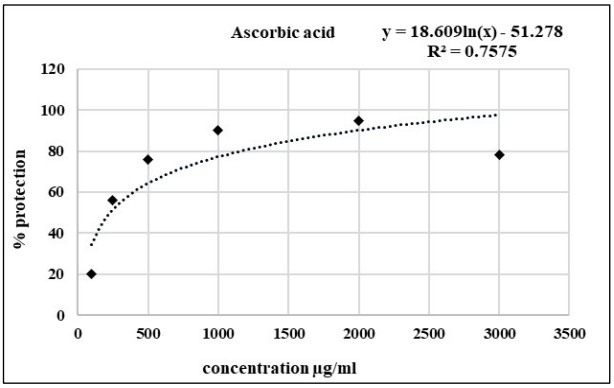


Figure 3. Anti-hemolytic activity of Ascorbic acid IC50 (µg/ml)



For normal erythrocytes, *C. iberica* extract showed an anti-hemolytic effect in a concentration-dependent manner. It provided notable activity at concentrations range of (2000-3000 µg/ml), in comparison to Ascorbic acid as a positive control, in terms of percentage inhibiting activity which ranged from 84.55% to 89.73% (table 2, figure 4). The IC50 value of *C. iberica* extract was 623.41µg/ml (figure 5). The lower the IC50 the more protection offered against hemolysis. Significant differences ($p<0.001$) between extract and Ascorbic acid were noticed at all tested concentrations.

Figure 4. The anti-hemolytic activity of *C. iberica* extract in comparison to Ascorbic acid

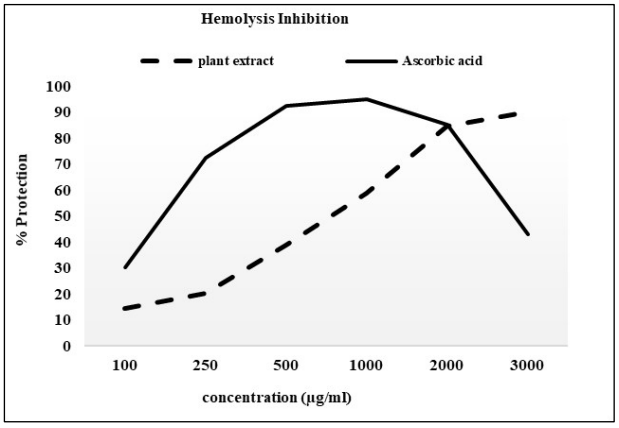
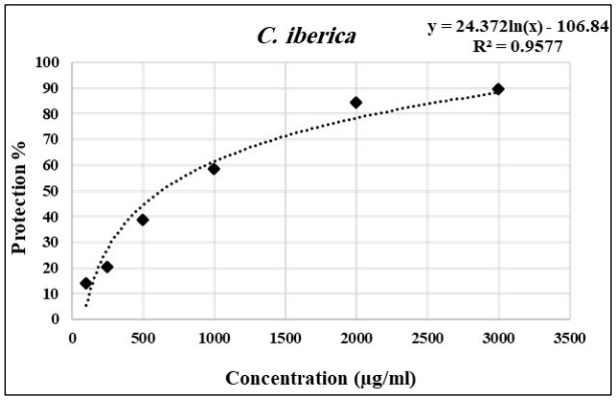


Figure 5. The anti-hemolytic activity of *C. iberica* IC50 (µg/ml)



In the case of G6PD-deficient erythrocytes, the extract also provided excellent activity with a maximum protection value of (92.72%) at the concentration (3000 µg/ml) (table 3).

Table 3. The protective effect of *C. iberica* extract for G6PD enzyme-deficient human erythrocytes

C. iberica		
Concentration (µg /ml)	Hemolysis%	Protection%
1000	10.28	89.72±0.9
2000	9.39	90.61±1.13
3000	7.28	92.72±1.3

Protection values are represented as mean ± SD

4. Discussion

In comparison with a previous study that used ultrasound-assisted extraction and was conducted in Turkey, the extraction yield of *C. iberica* using different solvents (chloroform, methanol, n-Hexane) was (1.46%, 7.04%, 1.14%), respectively. The difference in yield can be explained by different environmental factors (Erel *et al.*, 2014). In our study, the ultrasound-assisted extraction technique was used. As per to previous study (Albayrak *et al.*, 2017), ultrasonic extraction gave a better extract with a larger amount of extractive substances and stronger antioxidant and antimicrobial activities than the extracts prepared using other techniques (Soxhlet extraction, Maceration).

The result implied that *C. iberica* aerial parts hydromethanolic extract contains good phenolic content. Phenolic compounds are widely

found in plant products, and they are found to have antioxidant activities (Nabavi *et al.*, 2010), these activities due to their general structure which consists of hydroxyl group attach to the aromatic ring which is capable donating electron and stabilizing free radicals (Afsar *et al.*, 2016).

The increase in toxicity values with an increase in plant extract concentration is due to the quantity of chemical compounds contained in the extract. *C. iberica* aerial parts extract contains saponins as demonstrated by the results of the chemical analysis, which were known for their hemolytic effect (Noudeh *et al.*, 2010). With consideration that the color of the extract in high concentrations increases the absorbance value versus the colorless PBS solution.

The principle of this assay is that hydrogen peroxide generates radicals that attack the erythrocyte membrane and thus induce the chain oxidation of lipids and proteins, and cause hemolysis (Joshan and Rawal, 2012). Ascorbic acid was used in this test as positive control because it is well-known antioxidant agent, it may scavenge radicals and thus inhibit cytotoxicity prompted by oxidants (Boonkasem *et al.*, 2015). Our results are in agreement with the previous study which reported that low concentrations of Ascorbic acid provided good antioxidant activity, while high concentrations caused hemolysis (Ibrahim *et al.*, 2006). Our results suggest that *C. iberica* aerial parts extract is a good anti-hemolytic agent and offeres biological action compared with the standard used. This result reflects the antioxidant capacity of the extract which has been reported to be highly correlated with the content of phenolic compounds (Sariburun *et al.*, 2010). In this context, the inhibitory effect of the plant extract may be due to its phenolic compounds which can quench the radicals generated by H_2O_2, before these radicals attack the erythrocytes membrane and cause oxidative hemolysis.

5. Conclusion

According to the literature survey, this is the first report of the hemolytic and anti-hemolytic effects of *C. iberica*. The current study concluded that *C. iberica* aerial parts extract contains good levels of total phenolic compounds and also many bioactive constituents. The results demonstrated that *C. iberica* has a low hemolytic effect, and also showed appropriate anti-hemolytic activity against hydrogen peroxide-induced hemolysis. Therefore, it can be suggested that *C. iberica* aerial parts extract may have a beneficial effect in the treatment of oxidative stress-related diseases. Additional studies are wanted to support these results.

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