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PHYTOCHEMICAL STUDY AND PROTECTIVE EFFECT INVESTIGATION AGAINST OXIDATIVE DAMAGE IN HUMAN ERYTHROCYTES OF SYRIAN ASTERACEAE PLANTS

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Oxidative stress is a major cause of many diseases, it can damage the tissues and lead to lipid peroxidation and DNA strand breakage. Nowadays, researchers have centered medicinal plants in their concern, to benefit from their active component as antioxidant reagents. This study aims to investigate the protective and hemolytic activity, as well as qualitative and quantitative chemical content of plants from Syrian Flora. Ethanol 70% extracts of three plants of Asteraceae; Onopordum carduiforme, Centaurea verutum, and Achillea santolina were prepared to test their protective antioxidant effect on erythrocytes membrane against the oxidative hemolysis which was induced using AAPH. The hemolytic effect of these plants was also evaluated. In addition to the quantitative content of phenols TPC and flavonoids TFC compounds using Folin-Ciocalteu and aluminum chloride colorimetric methods respectively. The basic phytochemical screening was conducted using the standard methods.The results showed a low hemolytic effect of the extracts on erythrocytes, using the concentrations of (1-5 mg/ml). Moreover, they reduced membrane lipid peroxidation significantly; where O. carduiforme showed the highest efficacy of protective activity against hemolytic damage. The phytochemical screening of the plants' extracts showed phenols, flavonoids, tannins, coumarins, cardiac glycosides, terpenoids, steroids, and carbohydrates presence, whereas no alkaloids or saponins were found. TPC and TFC content ranges were between (9.22 ± 0.412 and 13.716 ± 0.431 mg GAE/g DW) and (2.224 ± 0.346 and 8.958 ± 0.216 mg RUE/g DW) respectively.

INTRODUCTION

Many disorders are caused by oxidative stress; these diseases may cause harmful effects on tissues or organs, which lead subsequently to lipid peroxidation and DNA strand breakage. The presence of hemoglobin, unsaturated fatty acids, and oxygen, may make erythrocytes more prone to oxidative damage, which cause membrane disruption and eventually hemolysis. Erythrocytes normally have a strong antioxidant activity, that includes enzymatic and non-enzymatic mechanisms, which act together efficiently to change ROS species into less reactive intermediate types. Unfortunately, these defense capabilities are limited, because mature erythrocytes lack the synthesis of anti-oxidative enzymes. Antioxidant agents such as vitamins C, E, and A can help in the prevention and treatment of many oxidative-mediated erythrocyte damages[1.]

Plants have been used by the public for thousands of years, all over the world and in

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many aspects. According to the World Health Organization, 80% of the world's population uses plant-based remedies as their primary form of healthcare. In some countries, such as in Europe herbal medicines have an ancient history and tradition[2]. Medicinal plants contain hundreds, if not thousands, of chemicals that interact in complicated ways, giving many bioactive effects. Despite that their traditional medicinal benefits are scarcely proven, some scientifically documented components are well-known to give the plants their medicinal and therapeutic properties, such as essential oils, phenolic compounds, alkaloids[3].

Onopordum carduiforme, *Centaurea* verutum, and Achillea santolina that represent the selected plants in this study belong to the Asteraceae family, which is one of the largest families in the plant kingdom, with an estimated number of 25,000 species spreading in wide areas. Additionally, this family comes second in Syria in terms of species figures (400 species in Syria). The Taxonomical classification of these plants is as follows: (kingdom: Plantae, Phylum: magnoliophyte, class: Magnoliopsida (Dicotyledons), order: Asterales, family: Asteraceae)[4-7].

Onopordum carduiforme is a biennial or perennial herb, 30-100 cm height, sparsely cobwebbed, stem erect, much-branched; wings consisting of triangular long-spiny lobes connected by a narrow green margin, leaves are almost glabrous on the upper face, often cobwebbed and greyish on the lower, florets dark purple[8]. *Onopordum* genus has been widely used in folkloric treatments, as refreshing, invigorating, and the treatment of inflammatory diseases and kidney problems[9-11]. Various studies have demonstrated the efficacy of antihypertensive, antioxidant, and antibacterial properties of other species of *Onopordum*[12-16]. These therapeutic properties are related to the wide range of secondary metabolites content, such as phenolic compounds, lignans, sesquiterpene lactones, coumarins, terpenes, and steroids[17].

Centaurea verutum is an annual plant, 50-130 cm height, stem rigid, erect, simple, or with erect branches; stem and leafy branches, lower leaves are mostly sinuate, other leaves are entire, oblong, the florets are yellow[8]. This plant was used as an expectorant, antidiabetic, antipyretic, and antidiarrheal in Turkey, and to treat infections, stomach ache, edema, arthritis, and pain in Northern Nigeria[18&19]. Several studies have confirmed that species of *Centaurea* have antibacterial and antioxidant properties[19&20].

Achillea santolina is a perennial herb, 15-30 cm height, woolly, stems erect to ascending, simple or branched, leafy up to the inflorescence, leaves narrow, linear, green[8]. In Syria, the genus of *Achillea* has been used traditionally for the treatment of gallbladder stones, anticancer, and atherosclerosis[21] and used to treat liver and kidney, digestive, cold, and high blood pressure diseases. Moreover, it has anti-inflammatory, antihelminthic, antiseptic for urinary tract infections, and anti-hyperglycemia properties[22&23]. Pharmacological studies have shown that *A. santolina* has a different chemical and therapeutic properties as an antioxidant and anticancer properties[24]. Other studies have showed the efficacy of antihypertensive, antidiabetic, and anti-hyperlipidemia[25&26]. In addition to that, it has a proven inhibitory activity on wide spectrum of bacteria species and leishmania[27].

Unfortunately, there are no sufficient scientific studies on Syrian Flora plants, especially in terms of investigating the biological activity. Furthermore, the Asteraceae members we chose have not received much attention in the literature and a little is known about these plants. As far as our knowledge is concerned, no research articles dedicated to these plants have been published in Syria of the same methodology aspects. Consequently, this study aimed to demonstrate the phytochemical constituents, determine the antioxidant compounds (phenols and flavonoids) of the three plants. In addition to conducting a biological study, that includes investigating the potential hemolytic effect of the plant towards human erythrocytes membranes and the protective antioxidant effect against the induced oxidative hemolysis that is caused using in vitro model of AAPH (2,2-azobis 2-amidinopropane dihydrochloride).

MATERIALS AND METHODS

Chemicals and Equipment

Chemicals: Ethanol GR (Eurolab, UK), Folin-ciocalteu phenol reagent (Sigma-Aldrich, Switzerland), Sodium Carbonate anhydrous (PAREAC QUIMICA SAU, Spain), Gallic acid (Titan biotech LTD., India), Rutin (ExtrasyntheseGenay, France), Aluminum Chloride Hexahydrate (Scharalau Chemie, Spain), Distilled deionized water (d.H2O), Triton- X100 (Roth, Germany), 2,2- azobis 2-amidinopropane dihydrochloride (Sigma-Aldrich, USA), Vitamin C

Equipment: Sensitive balance (Sartorius TE214, Germany), Rotary evaporator (Heidolph Instruments, Germany), UV-1800 spectrophotometer (Shimadzu, Japan), Ultrapure TM water purification system (Lotun Co., Ltd., Taipei, Taiwan), Centrifuge Germany, (HeraeusMegafuge), ultrasonic bath (POWERSONIC 405, Hwashin Technology Co., Korea), Disposable Syringes 5, 10 ml (UK), EDTA tub (China), Eppendorf Tubes.

Plant Material

Fresh aerial parts (stems, flowers, leaves) of *O. carduiforme, C. verutum, A. santolina* were collected from different areas of the Aleppo Governorate in Syria. Plant specimens were identified by Dr. Ahmed Jadouh, Professor and expert at the Faculty of Agricultural Engineering, Aleppo University, Syria. The aerial parts were washed under running tap water, shade dried, then powdered using a mechanical grinder and kept in an airtight glass container until use.

METHODS

Preparation of extracts

The powdered plant samples (130 g of each plant) were extracted by Ultrasonication Assisted Extraction, using ten folds of ethanol 70% at 40^{o}C for one hour. The extracts solutions were then filtered through Whatman No. 1 filter papers, and the residual material was re-extracted three times using the same procedure. The combined extracts were evaporated using the rotary evaporator at 40°C to remove the solvent. The crude extracts were kept separately in sterile sample tubes and stored at 4°C for further usage[28&29].

The yield percentage was then calculated using the following equation:

$$\text{Yield (\%)} = W_{ex}/W_p * 100$$

Where W_{ex} is the weight of the dried extract and W_p is the weight of the dried plant material[30].

Phytochemical screening

Phytochemical examinations were carried out for the plants' extracts as per the standard methods[31]. Three plants were screened for their phytochemical components of phenols, flavonoids, alkaloids**,** tannins, saponins, coumarins, cardiac glycosides, terpenoids, steroids, and carbohydrates[32-38]. (Table 1).

Table 1:: Phytochemical examinations for plants extract by using standard methods

Plants constituent	Test/Reagent used	Plants constituent	Test/Reagent used
Phenols	Ferric Chloride	Saponins	foam
	Folin Ciocalteu		aromatic aldehydes
Flavonoids	Aluminum chloride	Coumarins	fluorescence
	Shinoda test	Cardiac Glycosides	Killer-Killiani
	Pew's Tests		Kedde
Alkaloids	Dragendroff	Terpenoids	Salkowski test
	Mayer's		Libermann Burchard's
Tannins	Ferric chloride	Carbohydrates	Molisch's
	Lead acetate test		Fehling's
	Vanillin	Cyanogenic Glycosides	Picrate paper

Determination of total phenolic contents (TPC)

The total phenolic content of the plants' extracts was determined spectrophotometrically by using Folin-Ciocalteu's reagent. 2.5 ml of 10% Folin-Ciocalteu's reagent diluted in distilled water (d H_2O), was mixed with 0.5 ml of each extract solution of the concentration (0.5 mg/ml) in (ethanol 70%) solvent; the mixture was placed for few min, and then 2.5 ml of 7.5% Na_2CO_3 was added, the sample was incubated afterward at 45°C for 45 min. In the end, the absorbance was read using a spectrophotometer at λ_{max}= 765 nm against the blank solution which contains 0.5 ml solvent, 2.5 ml 10% F-C reagent, and 2.5 ml of 7.5% sodium carbonate. Each test was done in triplicate. The samples were independently prepared in triplicate for each analysis and the mean value of three absorbance was obtained. The same procedures were repeated for the standard solution of Gallic acid in distilled water (d H_2O) as a standard series (from 0.01 to 0.08 mg/ml) the liner calibration was construed. Based on the measured absorbance, the concentration of phenolic compounds was calculated from the equation of the calibration line. The content of phenolic in each extract was expressed in terms of milligrams of gallic acid equivalent per gram of plant dry weight or mg of gallic acid equivalent per gram of dry extract (mg GAE/g DW, mg GAE/g extract)[39].

Determination of total flavonoid content (TFC)

The content of flavonoids in the extracts of the examined plants was determined using the spectrophotometric method. The test sample contained 1 ml of (ethanol 70%) solution of the extract in the concentration of 1 mg/ml for *O. carduiforme*, and 0.5 mg/ml for both *C. verutum* and *A. santolina*, in addition to 1 ml of 2% $AlCl_3$ solution dissolved in methanol. The samples were incubated for an hour at room temperature. The blank sample consists of 1 ml extract solution with 1 ml methanol without $AlCl_3$. The absorbance was read using a spectrophotometer at λ_{max}= 415 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of rutin in ethanol (0.005 to 0.6 mg/ml) and the calibration line was construed. Based on the measured absorbance, the concentration of flavonoids was calculated from the equation of the calibration line. Then, the content of flavonoids in extracts was expressed in terms of milligrams of rutin per gram of plant or mg of gallic acid equivalent per gram of dry extract (mg RUE/g DW, mg RUE/g extract)[39].

Hemolysis test[40&41]

This test evaluates the effect of the plants' extracts on the red blood cells, to investigate whether they have a hemolytic effect or not, by using increased concentrations of the plants' extracts. Fresh human blood samples were taken from healthy volunteers being granted permission, non-smokers, with an average age of 25 years. The blood was collected in EDTA-containing tubes (Ethylene Diamine Tetra Acetic acid) as an anticoagulant, and centrifuged (at 2500 rpm for 10 min., at temperature (20-25°C). After that, the plasma was washed three times or more (with PBS 10 mmol pH = 7.4) until plasma and buffy coat removal. The red blood cells suspension (RBC) (10% - v /v) was prepared in (PBS). A mixture of 2.5 ml of 10% RBC suspension with 500 µL of the plants extract at the concentrations of (1,2,3,4 and 5) mg/ml prepared in (PBS) was gently shaken in a water bath at 37ºC for 40 min. Then, the tubes were centrifuged at 2500 rpm/min for 15 min. to allow broken membranes and unbroken cells to settle at the bottom. For positive control, or (100% hemolysis), 500 µL of 0.2% Triton in (PBS) was added to 2.5 ml of 10% RBC suspension. The supernatant was removed, and the liberated hemoglobin in the supernatant was measured using the spectrophotometric method for absorbance at 540 nm. For negative control, or (0% hemolysis), only 500 µL of PBS was added to 2.5 ml of 10% RBC suspension. The experiment was done in five replicates and the mean ± standard deviation was calculated as follow

% Hemolysis = (Absorbance of the sample/Absorbance of positive control) × 100.

Erythrocyte hemolysis induced by peroxyl free radical (AAPH)[40&42]

Fresh human blood samples were taken from healthy volunteers being granted permission, non-smokers, with an average age of 25 years. The blood was collected in EDTA-containing tubes as an anticoagulant, and centrifuged (at 2500 rpm for 10 min, at temperature (20-25°C). The plasma was washed three times or more (with PBS 10 mmol pH = 7.4) until plasma and buffy coat removal. The red blood cells suspension (RBC) (20% -v/v) was prepared in (PBS). Mixed 0.1 ml of 20% RBC suspension with 0.2 ml of 200 mM 2, 20 – azobis (2-amidinopropane) dihydrochloride (AAPH) solution (in PBS) and 0.1 ml of extract of the plants at concentrations (1,2,3,4,5) mg/ml Prepared in (PBS). The reaction mixture was shaken gently (30 rpm) in a water bath at 37ºC for 3 hours. Then they were centrifuged at 5000 rpm/min for 10 min. 0.3 ml of supernatant liquid diluted with (0.6 ml) of PBS and using the spectrophotometric method for absorbance at (540 nm). The positive control is AAPH and red blood cell suspension without the plants' extracts. The negative control is PBS and red blood cell suspension. L-ascorbic acid was used as a reference standard. The experiment was done in five replicates and the mean ± standard deviation was calculated as follow

% hemolysis inhibition = $[(AAPH_{AS})/AAPH_{+c}].100$, where AS is the absorbance of the sample, and +c is the absorbance of positive control.

RESULTS

Extraction yield

The crude dried extracts obtained were nicely aromatized, brown, and the yield percentage of the solid residue weight of *C. verutum* extract was higher than the others plants, followed by *O. carduiforme* extract and *A. santolina* extract came last in yield percentage, as shown in (Table 2).

Table 2: The yields percentage of solid residue.

Plants	Yield %
Onopordum carduiforme	13.44%
Centaurea verutum	15.92%
Achillea santolina	9.17%

Phytochemical analysis

Fundamental phytochemical screening results showed that the hydroethanolic extracts of the three plants contain all of the following phytochemical groups; phenols, flavonoids, tannins, coumarins, cardiac glycosides, terpenoids, steroids, and carbohydrates. Whereas no alkaloids or saponins were found (Table 3).

Total phenolic content (TPC)

The total phenolic content of the examined plants' extracts was calculated according to the equation of calibration curve for gallic acid (Figure 1), (y= 5.9333x + 0.0324, R^2= 0.9988). The values obtained for the extracts content of total phenols were expressed as mg of GAE/g of plants dry weight. The results range was between (9.22 ± 0.412 and 13.716 ± 0.431 mg GAE/g DW), and the highest content was recorded by (*C. verutum*) (Table 4).

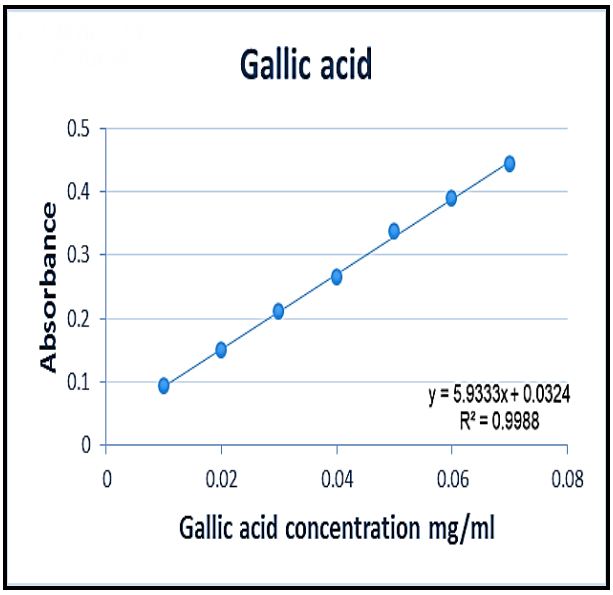

Fig. 1: Calibration curve of Gallic acid for determination of TPC.

Table 3: Phytochemical screening of various extractives of *O. carduiforme*, *Centaurea verutum* and *Achillea santolina*

Plants constituent	Test/Reagent used	*Onopordum carduiforme*	*Centaurea verutum*	*Achillea santolina*
Phenols	Ferric Chloride	+	+	+
	Folin Ciocalteu	+	+	+
Flavonoids	Aluminum chloride	+	+	+
	Shinoda test	+	+	+
	Pew's Tests	+	+	+
Alkaloids	Dragendroff	-	-	-
	Mayer's	-	-	-
Tannins	Ferric chloride	+	+	+
	Lead acetate test	+	+	+
	Vanillin	+	+	+
Saponins	foam	-	-	-
	aromatic aldehydes	-	-	-
Coumarins	fluorescence	+	+	+
Cardiac Glycosides	Killer-Killiani	+	+	+
	Kedde	+	+	+
Terpenoids	Salkowski test	+	+	+
Steroids	Libermann Burchard's	+	+	+
Carbohydrates	Molisch's	+	+	+
	Fehling's	+	+	+
Cyanogenic Glycosides	Picrate paper	-	-	-

+ Present, - absent

Table 4: Total phenol content (TPC) in the plants extracts.

Plants	TPC (mg GAE/g DW)	TPC (mg GAE/g Extract)
Onopordum carduiforme	10.301 ± 0.351	84.906 ± 2.892
Centaurea verutum	13.716 ± 0.431	97.257 ± 2.610
Achillea santolina	9.193 ± 0.412	105.675 ± 4.740

GAE: gallic acid equivalents

Each value is the average of three replicates ± standard deviation

Total flavonoids content (TFC)

The total flavonoid content of the plants' extracts was determined using the aluminum chloride colorimetric method, and calculated according to the equation of calibration curve for rutin (Figure 2), ($y= 14.682x + 0.0213$, R^2 0.9992). The results were expressed as mg of RUE/g of plant's dry weight. While (*C. verutum*) scored the highest content, the whole results ranged between (2.224 ± 0.346 and 8.958 ± 0.216 mg RUE/g DW). (Table 5).

Table 5: Total flavonoids contents (TFC) in the plants extracts.

Plants	TFC (mg RUE/g DW)	TFC (mg RUE/g Extract)
Onopordum carduiforme	2.224 ± 0.346	17.097 ± 0.927
Centaurea verutum	8.958 ± 0.216	62.211 ± 1.499
Achillea santolina	5.183 ± 0.231	59.578 ± 2.65

RUE= Rutin equivalents

Each value is the average of three replicates ± standard deviation

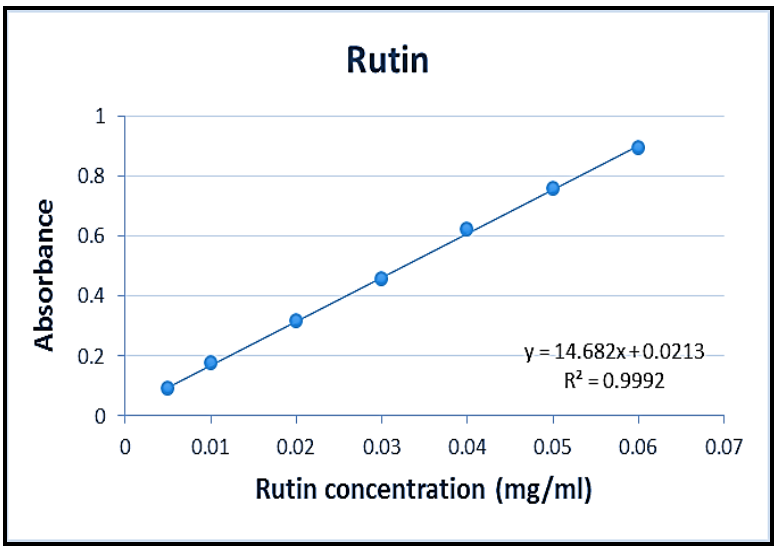

Fig. 2: Calibration curve of Rutin for determination of TFC

Hemolysis test

A hemolytic assay experiment was followed to evaluate the effect of *O. cadruiforme*, *C. verutum* and *A. santolina* extracts on red blood cells. The hemolysis test result showed that the plants' extracts have caused hemolysis percentages that are close to the negative control effect (Table 6, Figure 3). There was a clear relationship between the increased plant concentrations and the measured absorbance values, which represent the hemolysis action. At the highest concentrations used (4,5 mg/ml) the hemolysis percentage was slightly more observed, but still not statistically significant as compared to the negative control. Thus, we can consider these plants' extracts as safe and nontoxic to red blood cells in the used concentrations.

Table 6: The percentages of hemolytic effect by *O. carduiforme, C. verutum , A. santolina* extract and Negative control:

Plants' extracts	Hemolysis%				
	Concentration (mg\ml)				
	1	2	3	4	5
O. carduiforme	7.216 ± 2.13	7.518 ± 2.04	7.772 ± 2.14	8.15 ± 2.00	9.44 ± 1.89
C. verutum	7.034 ± 2.14	7.364 ± 2.16	8.014 ± 2.05	8.998 ± 1.74	10.236 ± 1.78
A. santolina	6.77 ± 2.51	7.088 ± 2.76	8.062 ± 3.07	8.18 ± 2.78	9.228 ± 2.90
Negative control control (PBS buffer)	5.948				

Each value is the average of five replicates ± standard deviation

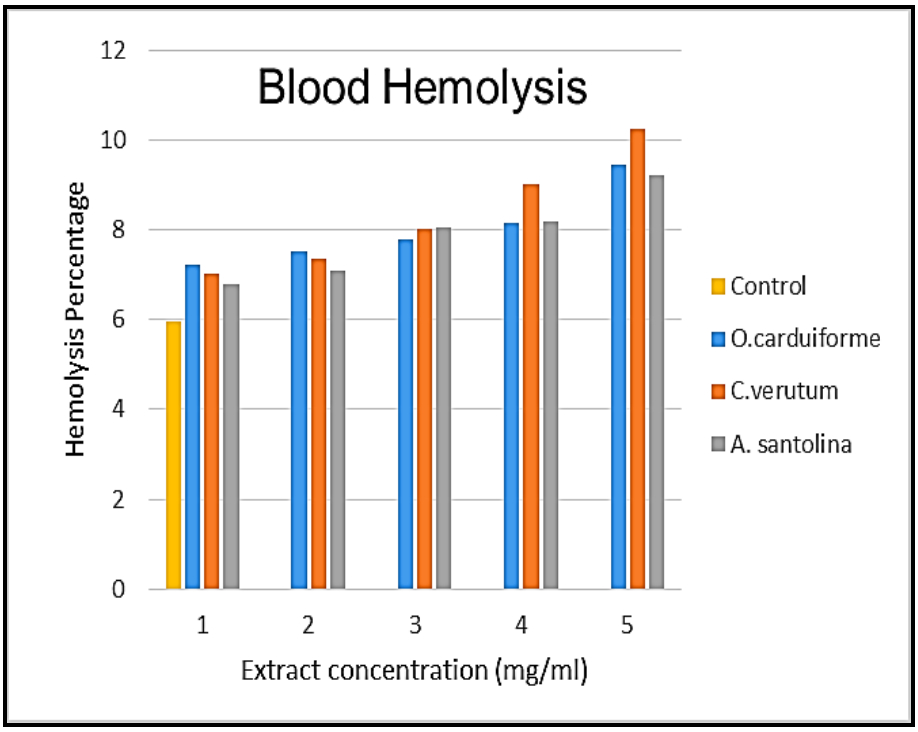

Fig. 3: The percentages of Hemolysis blood of plants extract in comparison with negative control

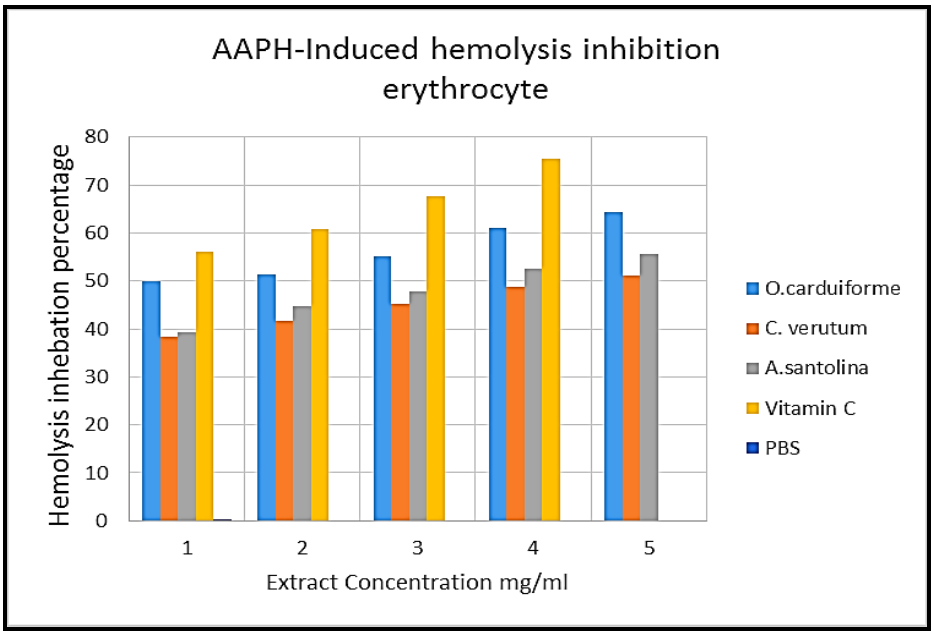

Fig. 4: Hemolysis inhibition (%) of the plants extract extracts in comparison with vitamin C.

Erythrocyte hemolysis induced by peroxyl free radicals (AAPH)

The oxidative hemolysis in erythrocytes induced by AAPH (2,2- azobis 2-amidinopropane dihydrochloride) has been by oxidative stress in the erythrocyte membranes. extensively studied as a model for peroxidative damage in bio-membranes. This study evaluates the ability of studied the plants' extracts to reduce the damage caused

Table 7: The percentages of Protection hemolysis% effect by *O. Carduiforme, C. verutum, A. santolina* extract and Vitamin C

Plants' extracts	Protection hemolysis%				
	Concentration (mg\ml)				
	1	2	3	4	5
O. carduiforme	49.988 ± 20.98	51.416 ±2 2.54	55.158 ± 21.70	61.03 ± 20.92	64.332 ± 20.45
C. verutum	38.358 ± 198	41.636 ± 14.98	45.232 ±11.26*	48.7 ± 11.64 *	51.148 ± 4.96
A. santolina	39.39 6± 19.64	44.752 ± 19.74	47.87 ± 6.57*	52.45 ± 20.39 *	55.702 ± 8.95
Vitamin C (positive control)	56.106 ± 7.68	60.744 ± 7.38	67.76 ± 8.07*	75.84 ± 6.21 *	—
PBS (Negative control)	0.062 ± 0.024				

*Express the presence of a significant difference with Vitamin C at Significant differences at ($P < 0.05$)

Each value is the average of five replicates ± standard deviation

The results showed that all of the plants' extracts are effective as they can inhibit oxidative hemolysis (Figure 4). The percentage range that represents the reduction of oxidative hemolysis was between (38.358 ± 19.80% and 64.332 ± 20.45%). O. carduiforme showed the highest protective effect against erythrocytes oxidative hemolysis (49.98% at 1 mg/ml) and (64.32% at 5 mg/ml). While C. verutum has the lowest protective effect against erythrocytes hemolysis (51.14% at 5 mg/ml). The percentages of hemolytic effect for all the plants' extracts were significantly higher than the negative control. However, the inhibition percentage of the standard L-ascorbic acid on hemolysis of red blood cells was (56.106 ± 7.68 at 1 mg/ml). While at 5mg/ml L-ascorbic acid showed erythrocytes hemolysis and no protective effect. Significant differences between extract and positive control are represented in (Table 7).

DISCUSSION

Phytochemical screening

The phytochemical analysis of plants is an essential process in each phytochemical study. The chemical compounds that have been found in the studied plants are known to provide important biological activities, which gives these species their medical importance. Phytochemical screening results showed that the hydroethanolic extract contains phenolic compounds, flavonoids, tannins, coumarins, cardiac glycosides, terpenoids, steroids, and carbohydrates. The results of *O. carduiforme* were similar to the results of another study of *O. macrocephalum* which have been studied in Aleppo University too. With one exception, that *O. macrocephalum* does not have coumarins[16]. While another phytochemical study of *Onopordum acanthium L*. showed that it has coumarins in its aerial parts[43]. The results for *C. verutum* were consistent with the previous study of *Centaurea cineraria L.* as both species have carbohydrates, phenols, tannins, and flavonoids, but do not have saponins[44]. However, *Centaurea ammocyanus* has a little amount of saponins[45]. *Achillea santolina* contains phenolic and flavonoids compounds, and this matches what Al-Snafi has reported in his results[46]. Tannins, steroids, and terpenoids were found in *Achillea tenuifolia LAm* extract[47]. However, these agreements/disagreements among studies and the plants included are typically normal, and may be due to the difference in plant parts used as raw material, the solvent used for extraction, or the extraction procedure[31]

Total phenolic content

Phenols form the largest group of plant secondary metabolites widespread in nature, and they are very important compounds of medicinal plants. At first, it should be noticed that the phenolic content in one plant species, in general, is affected by several parameters, such as the solvent used to prepare the plant extract, the environmental conditions of each geographical area, and the collecting time of plant samples[44].

The highest phenolic content in this study was observed in *C. verutum* (13.716 ± 0.431 mg GAE/g DW), followed by *O. carduiforme* (10.301 ± 0.351 mg GAE/g DW), and the lowest was in *A. santolina* (9.193 ± 0.412 mg GAE/g DW).

The result for phenolic content of *O. carduiforme* in this study exceeds the ones of Sharif *et al* of other *Onopordum* species from Syria, AqME* extracts of *Onopordum macrocephalum* where TPC was reported as (8.15 ± 0.35 and 8.87 ± 0.19 mg GAE/g DW) for floral parts and vegetative parts respectively[14]. The TPC of *O. carduiforme* was (84.906 ± 2.89 mg GAE/g extract) which is slightly lower than *Onopordum acanthium* (89.3 ± 0.13 mg GAE/g extract)[48]. The TPC of Aq extract of *Centaurea cyanus L.* was (5.65 mg GAE/g DW)[49] while in another study in Syria of AqME extract of *C. cineraria* the TPC was (41.12 ± 0.6 mg GAE/g extract). Therefore, it can be concluded that the species of *Centaurea* in our study have a higher content of phenols[44].

Achillea santolina has the lowest content of phenols in this study among the three Asteraceae plants (105.675 ± 4.74 mg GAE/g extract). However, it exceeds other species like *Achillea sivasica* whose TPC was (34.5 ± 1.7 and 51.1 ± 0.8 mg GAE/g extract) for herbal and floral parts respectively[50]. In addition, our results are in resemblance with Ardestani's, which showed TPC of *A. santolina* as (104.66 ± 4.39 mg GAE/g extract)[51]. These differences in the contents might be because of polar phenolic hydroxyl group/s substitutions in phenol structure or their high extraction tendency into polar solvents. However, ethanol 70% solvent seems to possess higher concentrations of bioactive phenol compounds, and this could be related to polarity that tend to dissolve easily in such solvent[31&52].

Total flavonoids content:

Flavonoids which occur both in the free situation and as glycosides, are the largest group of naturally occurring phenols. More than 2000 of these compounds are well-known and have already been isolated, 500 of them exist in the free state[53]. The highest flavonoid content in this study was observed in *C. verutum* (8.958 ± 0.216 mg RUE/g DW), followed by *A. Santolina* (5.183 ± 0.231 mg RUE/g DW) and the lowest was found in *O. carduiforme* (2.224 ± 0.346 mg RUE/g DW). The Flavonoids content of *O. carduiforme* was lower than *Onopordum macrocephalum* floral parts and vegetative parts, where TFC was reported as (14.35 ± 0.0.19 and 12.35 ± 0.61 mg RUE/g DW) respectively[14]. The TFC of *Centaurea verutum* was (62.211 ± 1.49 mg RUE/g extract), and thus it is higher than other species; *C. kotschyi var. persica* and *C. tchihacheffii*, where the TFC was (5.76 ± 1.65, 3.06 ± 0.55 mg RUE/g extract) respectively[54].

Finally, the TFC of *Achillea santolina* was (5.183 ± 0.231 mg RUE/g DW), and *A. schurii* extract was (38.61 ± 2.39 mg RUE/g DW)[55]. These figures variation may be related to the polarity of solvents. Interestingly, it is important to emphasize that the TFC is lower than TPC, supporting the fact that flavonoids belong to the phenols major group. Moreover, the differences in total phenols and flavonoids content among species could be due to several intrinsic and extrinsic factors, such as the genetic potential of individual species for polyphenol biosynthesis, the environment and maturation stage of each plant may also be critical in this aspect[39&52].

Hemolysis test

A toxicity test is so important in investigating the effect of natural compounds on the red blood cell membrane; hemolytic activity assessment is useful for this purpose. This test has been widely used as a model for studying the interaction of drugs with cellular membranes. It presents a direct indication of the toxicity of injectable formulations[44].

The percentage range of hemolysis that was affected by the plants was between (6.77 ± 2.51 and 10.236 ± 1.78%). Statistically, no significant effect was found between red blood cells that were treated with the extracts compared to the negative control.

It is interesting also to note that, the color of the extract itself in its highest concentrations (4 - 5) mg/mL might have caused an additional increase in the measured absorbance value against the colorless physiological solution. Where the percentage of hemolysis is calculated as a result of the division: absorption of the sample containing the extract/absorption of the positive control (represents 100% hemolysis).

Some of the studies about the *Centaurea* genus showed that hemolysis percentage was 4.84 ± 0.9% of *C. cineraria* at 4000 μg/ml and this value was considered as not toxic on RBC[44]. Another Centaurea specie *C. ammocyanus* showed safe application without any significant difference compared to negative control in low concentrations (100-500 μg/ml), although this specie contains a little amount of saponins. However, at higher concentrations (1000-3000 μg/ml) the hemolysis percentage increased. Other plants from the Asteraceae family which contain little amount of saponons, such as *Sonchuso leraceus* and *Matricaria chamomilla*, possesed a low hemolytic effect on RBC in both methanolic and aqueous extracts[57&58]. The methanolic and aqueous extracts of *Silybum marianum* also contain saponins, and the hemolysis percentage at 1000 μg/ml was (42.7 ± 0.66, 36.5 ± 0.67%) respectively. To sum up, higher damage in RBC membrane is expected along with the higher presence of saponins. Fortunately, the Phytochemical screening (Table 2) shows that all of the three plants in this research do not contain saponins. However, some studies considered that plants' extracts are safe and do not show toxicity on red blood cells when the hemolysis percentage is less than 10%[59].

Saponins have potent toxicity by inducing hemolysis of red blood cells. The release of hemoglobin from erythrocytes is a result of changes in cells' membrane permeability. This is expected to be influenced by the affinity of the aglycone to cholesterol in cell membranes. The type of saponins and the quantitative content of the plant may cause different levels of hemolytic activity[56.] It should be noted that saponins are surface-active agents, with soap-like properties and can be detected by their ability to cause foaming or their ability to lyse blood cells. Another major reason for hemolysis is oxidative stress. The oxidative damage can affect proteins and lipids within the erythrocyte membrane and contribute to some hemoglobinopathies and lipid peroxidation[1&38]. In this study, the plants' extracts contain phenols, flavonoids, and tannins which are known to have antioxidant properties, so it can be considered that the studied plants are good protective agents against oxidative stress by their polyphenols content.

Erythrocyte hemolysis induced by peroxyl free radicals (AAPH)

Several tests are used in modern research to evaluate the extracts inhibition activity of hemolysis, such as the assessment by using hypotonic solutions, the assessment by using H_2O_2, or by peroxyl free radicals.

Centaurea cineraria and *Centaurea ammocyanus* have provided a significant protection against H_2O_2 induced oxidative damage in human erythrocytes at high concentrations (1000-4000 μg/ml) and (1000-3000 μg/ml) respectively[45&48].

Turkish *Achillea* specie which were used in traditional medicine were also studied. The results showed that all infusions of *Achillea* species were found to be protective against lipid peroxidation (LPO) levels of erythrocytes and leucocytes counter to H_2O_2 induced oxidative damage. In that study, *Achillea millefoliums sppannonica* and *Achillea falcata* (TFC % 0.250 ± 0.080, TPC % 157.78 ± 1.36 mg GA eq/l) had the lowest LPO levels on erythrocytes.

Achillea nobilissubspsipylea and *Achillea setacea* (TFC% 0.096 ± 0.015 TPC% 121.81 ± 1.82 mg GA eq/l) showed the highest protective effects on leucocytes[60]. *Sonchuso leraceus*, *Matricaria chamomilla*, and *Silybum marianum* from the Asteraceae family have

efficacy as an antioxidant via the inhibition of H_2O_2 induced hemolysis[56-58].

In this study, the assay for erythrocyte hemolysis was mediated by peroxyl free radical which is AAPH (2,2-azobis 2-amidinopropane dihydrochloride). AAPH is a peroxyl radical initiator that generates free radicals by its thermal decomposition, and then it attacks the erythrocytes to induce the oxidation chain of the lipids and proteins, disturbing the membrane structure and eventually leading to hemolysis[42].

The range of hemolysis inhibition percentage was between (38.358 ± 19.80 and 64.332 ± 20.45%), these values are similar to flowers extracts of *Cosmos sulphureus* plant (Asteraceae family), which ethyl acetate and acetone extracts were tested for their anti-hemolytic activity (30.12 ± 0.12 to 65.48 ± 0.26%) and (25.78 ± 0.10 to 62.92 ± 0.25%) respectively. These values have a correlation proportional to the gradual increase in the concentrations used. The result of the total phenols and flavonoids content showed that the extracts that contain a good amount of these phytochemicals, are more likely to show antioxidant properties. This difference in activity is related to variations in the quantity and chemical structure of phenols and flavonoids. As well as the substitution of hydroxyl groups on the phenolic aromatic ring, their hydrogen donor ability and forming resonance-stabilized phenoxyl radicals, as the antioxidant activity increases by increasing the degree of hydroxylation[52]. In this test, we used ascorbic acid as a positive control because it is well-known as an antioxidant agent. The results showed that the percentage of ascorbic acid protection of hemolysis activity at concentrations (1.2.3,4 mg/ml) was between (56.106 ± 7.68 and 75.84 ± 6.21%) %, but, at 5 mg/ml concentration, erythrocyte hemolysis was occurred, or its antioxidant efficacy was decreased, which suggested that, vitamin C might have a pro-oxidant activity. This is confirmed by several studies, which reported that there are some conditions when vitamin C can act as pro-oxidant, such as its high concentrations, the redox potential of the cellular environment (oxidises/redosis), and the presence or absence of transition metals. The pro-oxidant activity of vitamin C could be due to a potential reaction between vitamin C and transition metal ions such as free iron so that promote their reduction, increase H_2O_2 production and consequently $OH^{\cdot}$ formation of leading to oxidative damage to biomolecules or may interact with erythrocytes causing lipid peroxidation of the membrane, oxidation of hemoglobin and thus hemolysis[44&45,61&62].

Conclusions

The results of this study of three plants of the Asteraceae family from Syrian flora conclude that; *Onopordum carduiforme*, *Centaurea verutum*, and *Achillea santolina* are rich in phytochemical compounds especially phenols and flavonoids. The highest one in the total phenols and flavonoids contents was *Centaurae verutum*. All of the plants' extracts have very low hemolytic toxicity against human erythrocytes, have significant protective activity against lipid peroxidation, and could prevent oxidative damage induced by AAPH in normal human erythrocytes. The most protective extract was *Onopordum carduifome*. This study supports the theory that these plants can be a good source of natural antioxidants, that can be used in drugs industry as natural sources of antioxidants compounds in the case of oxidative diseases.

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دراسة كيميائية نباتية واستقصاء التأثير الواقي ضد الضرر التأكسدي لكريات الدم الحمراء البشرية لنباتات من الفصيلة المركبة السورية

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يسبب الإجهاد التأكسدي العديد من الأمراض عن طريق إتلاف الأنسجة والأعضاء، كما أنه يسبب أكسدة الليبيدات وتحطيم الحمض النووي DNA. لذلك في وقتنا الحالي يهتم الباحثون في النباتات الطبية للاستفادة من مكوانتها المضادة للأكسدة.

تهدف الدراسة الحالية في التحري عن التأثير الانحلالي و الفعالية الواقية للانحلال والكشف عن المكونات الكيميائية وتحديد المحتوى الفينولات والفلافونوئيدات للخلاصة الايتانولية المائية ٣٠:٧٠ % لثلاث نباتات من الفصيلة المركبة : Centaurea verutum, Onopordum carduiforme و Achillea santolina حيث تم اختبار التأثير المحتمل للخلاصة النباتية على كريات الدم الحمراء وتقييم نشاطها المضاد للانحلال من خلال دراسة التأثير الواقي لأكسدة الأغشية الخلوية. واعتماد الطرق الطيفية في تحديد المحتوى الكمي للفينولات والفلافونوئيدات حيث استخدم كاشف (Folin-Ciocalteu and aluminum chloride) على الترتيب. بالإضافة لإتباع طرق مرجعية في تحديد المكونات الكيميائية.

بينت النتائج أن الخلاصة النباتية لها تأثيراً حالاً منخفضاً عند التراكيز (١–٥ مجم/مل)، كما أنَها خفضت من أكسدة الأغشية الخلوية بشكل ملحوظ. حيث تبين أنّ لنبات Onopordum carduiforme الفعالية الأكبر في تثبيط الانحلال التأكسدي. كما أنها تحتوي على (فينولات، فلافونوئيدات، تانينات، كومارينات، غليكوزيدات قلبية، تيربونوئيدات، ستييروئيدات و كاربوهيدرات دون أن تحتوي على القلويدات والسابونينات). وبالنسبة لمحتوى كل من الفينولات والفلافونوئيدات فتراوحت نتيجة المحتوى بين (٩.٢٢ ± ٠.٤١٢ إلى ١٣.٧١٦ ± ٠.٤٣١ mg GAE / g DW) و (٢.٢٢٤ ± ٠.٣٤٦ إلى ٨.٩٥٨ ± ٠.٢١٦ mg RUE / g DW) على الترتيب. تستنتج الدراسة أنّ للخلاصة الإيتانولية المائية ٧٠:٣٠ % للنباتات المدروسة فعالية مضادة للانحلال التأكسدي وليس لها تأثيرات سمية على خلايا الدم الحمراء.