



Phytoconstituents, Free Radical Scavenging Potential, Total Phenols and Total Flavonoids Assessments for Violet Horned Poppy from Jerusalem Mountains

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Abstract

Most of the world population especially in the developing countries used herbal medicaments due to their cultural acceptability, safety, efficacy and fewer side effects for their primary health care. Dearth in the studies related to *Roemeria hybrida* has prompted us to do the present work. This study was designed to detect the presence of bioactive compounds and to evaluate total phenols, total flavonoids contents and antioxidant activity of *Roemeria hybrida* plant growing in the mountains of Jerusalem/ Palestine. The results of such a study would be important to prove or disprove its utilization in the Palestinian folk medicine for treatment of retinopathy (diabetes mellitus) and other diseases. Total flavonoid contents were determined by using Rutin reference standard method and total phenols determined by using Folin Ciocalteu's method while antioxidant activity evaluated by using 2,2-diphenyl-1-picryl-hydrazyl-hydrate assay. The results showed that the aqueous extract contains carbohydrate, reducing sugars, glycosides, flavonoids, terpenoids, phenols and tannin. The result also revealed that this plant has an antioxidant activity ($IC_{50}=31.405\pm 0.21\mu\text{g/ml}$), as well as total phenol was 30.448 ± 0.56 mg of GA/g while the total flavonoid was 53.821 ± 0.42 mg rutin/g of the plant extract. Therefore *R. hybrida* can be a good candidate for manufacturing of pharmacological active pharmaceuticals, cosmeceuticals and nutraceutical formulations.

Keywords: *Roemeria hybrida*; Phytochemical screening; Antioxidants; Total flavonoids, Total phenols.

1. Introduction

In the past few decades, there were huge advancements in the utilization of medicinal natural products in Europe, America, as well as people all over the world. This articulated a considerable interest and even an increased preference for their pharmaceutical forms and many names were given to these products such as botanical medicines, folkloric medicines, herbal medicinal products, herbal remedies, herbal medicines and traditional medicines [1, 2].

Now a day's, dietitians, physicians, nurses, pharmacists and other health professionals are being faced with situations in which their patients are using a huge and various numbers of herbal formulations and some of these herbal natural products have been placed in the food stores in various forms as tablets, capsules, tinctures, herbal teas and other forms to treat different health conditions [3, 4].

Antioxidant phytoconstituents that occur naturally in many herbal foods and produced by the body, work in the body together to preserve human health by protecting human cells from the harm caused by free radicals, which can damage healthy human tissues and cells, causing various conditions as arthritis, aging, atherosclerosis, cancer, cataracts, diabetes, pulmonary dysfunction, hemodialysis, pancreatitis, colitis, multiple sclerosis, Parkinson's disease, neonatal lipoprotein oxidation and skin lesions [5-8].

Roemeria hybrida (L.) DC. synonym Latin scientific name is *Chelidonium hybridum* L. belongs to the Papaveraceae family (English common name Violet Horned Poppy) which botanically described as annual herbaceous plant, about 20 cm tall, more or less hispid with 2 or 3 pinnatisect, 2-5 cm long leaves; petiole of the lower and basal leaves 1-2.5 cm long, somewhat broad and sheathing at the base; ultimate segments linear or ovate-oblong, hispid or hispidulous, without or with a pointed bristle at the apex. The flowers petals have a violet with a black blotch at base color, obviate to sub-rounded shape while the stamens are multi-seriate with subulate filaments while the seeds are rutted and reniform [9-11], as shown in Fig. 1.



Figure 1: Fresh and dried *R. hybrida* plants

The *Roemeria hybrida* plant is widely and wildy distributed in the Northern regions of Africa and in the Southwestern regions of Asia and Europe [12, 13]. The chemical composition of *Roemeria hybrida* has been investigated in various studies and some phytochemical constituents were recognized such as gossypetin 3-glucuronide-8-glucosides, herbacetin glycosides [14] and α -roemehybrine, roemerialinone, 8,9dihydroisoroemerialinone, roehybrine, roehybramine β -N-oxide, roehybridine α -N-oxide, 11,12-dihydroorientalinone, isoorientalinone proaporphine alkaloids [15, 16].

R. hybrida plant used traditionally in the Arab world specially by ethnocultural groups like Bedouins for treatment of various eye and vascular diseases especially for retinopathy and glaucoma (used as ophthalmic solution) also used for treatment of varicose veins and hemorrhoids where the aerial part of the plant prepared by boiling them with water for two hours then filtered and used regularly for two weeks [17, 18].

This plant used in the Palestinian and other Arabian folk medicines for treatment of retinopathy, glaucoma (as ophthalmic solution), varicose veins and haemorrhoids [19, 20].

2. Materials and methods

2.1. Collection and preparing plant materials

The aerial parts of *Roemeria hybrida* plant were collected in May, 2014 from Jerusalem Mountains (Alskehk Jarah and Abu kber)/Palestine. The plant was botanically identified by Dr. Nidal Jaradat from the Pharmacy Department at An-Najah National University. Voucher specimen was deposited in the Herbarium of the Pharmaceutical Chemistry and Technology Division (Laboratory of Pharmacognosy) and the *R. hybrida* plant herbarium voucher code is (Pharm-PCT-2044).

The plant aerial parts (flowers, leaves and stems) were gently washed and then dried in the shade at controlled temperature (25 ± 2 °C) and humidity (55 ± 5 RH), until all the plants parts became well dried. After that, the dried plant materials were powdered well by using mechanical grinder and placed into a well closed glass containers for further use.

2.2. Instrumentation

Shaker device (Memmert shaking incubator, Germany), rotary evaporator (Heidolph OB2000 Heidolph VV2000, Germany), spectrophotometer (Jenway 7135, England), freeze dryer (Mill rock technology, model BT85, Danfoss, china), grinder (Moulinex model, Uno, China), balance (Rad wag, AS 220/c/2, Poland), Automatic Deionizer Unit, Mime water inc. Haifa), filter paper (Machrery-Nagel, MN 617 and Whatman no.1, Sigma-Aldrich, United States).

2.3. Chemical Reagents

The following reagent were used in evaluations of total phenols, total flavonoids contents and antioxidant activity: Methanol (Iobachemie, India), n- hexane (Frutarom LTD, Haifa), Trolox ((s)-(-)-6 hydroxy -2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) (Sigma-Aldrich, Denmark), (DPPH) 2, 2-Diphenyl-1-picrylhydrazyl (Sigma-Aldrich, Germany). Folin-Ciocalteu reagent (Sigma Aldrich, Denmark), Rutin hydrate (MP-Biomedical, USA), Millon's reagent (Gadot), Ninhydrin solution (Alfa Agar, England), Benedict's reagent (Gadot), Molish's reagent, H₂SO₄, iodine solution (Alfa aesar, England), NaOH (gadot), chloroform HCl (Sigma Aldrich, Germany) magnesium ribbon, acetic acid (frutarom LTD, Haifa), FeCl₃ (riedeldehan, Germany).

2.4. Preparation of plant extracts for phytochemical analysis

The aqueous extraction was performed by taking five grams of the plant powder and mixed with 200 ml of deionized water in a beaker. The mixture was heated on a hot plate at 30-40 °C and mixed with continuous stirring for 20 minutes. The mixture was filtered using Whatman filter paper no.1 and the filtrate was used for the further phytochemical analysis.

2.5. Preparation of plant extracts for total phenols, total flavonoids and antioxidant tests

About 10 g of the grounded aerial parts of *Roemeria hybrida* were soaked in 1 Liter of methanol (99%), placed in a shaker device at 100 rounds per minute for 72 hours at room temperature, and then stored in refrigerator for 4 days. After that, the extract was then filtered using Whatman filter paper no.1 and concentrated under vacuum on a rotator evaporator. The crude extract was stored at 4 °C in the refrigerator for further use.

2.6. Antioxidant activity

2.6.1. Trolox equivalent antioxidant plant activity

1,1-Diphenyl-2-picrylhydrazyl (DPPH), a stable free radical with a characteristic absorption at 517 nm, was used to study the radical scavenging effects of the extracts. As antioxidant donates protons to these radicals, the absorption decreases. The decrease in absorption is taken as a measure of the extent of radical scavenging [21].

2.6.2. Plant extracts

Series concentration of plant stock solution from 10 mg /100 ml methanol was prepared, then 1 ml of each concentration mixed with 1 ml of methanol with 1 ml of 0.002% DPPH, and all samples were incubated in a dark place for 30 minutes at room temperature, then the absorption was recorded at $\lambda_{\max} = 715$ nm.

1:1 methanol: DPPH blank solution was taken, and its absorbance was taken under consideration, to be used in calculating the percentage of inhibition.

2.6.3. Trolox standard curve

Concentration series of Trolox stock solution from 10 mg /100 ml methanol was prepared, then 1 ml of each concentration mixed with 1 ml of methanol plus 1 ml of 0.002% DPPH, and all samples were incubated in a dark place for 30 minutes at room temperature, then the absorption was record at $\lambda_{\max} = 715$ nm.

The working solutions of the following concentrations (1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80, 100µg/ml) were prepared by suitable dilution with methanol from the stock solution for the Trolox and for the plant.

2.6.4. Spectrophotometric measurements

DPPH was freshly prepared at a concentration of 0.002% w/v. The DPPH solution was mixed with methanol and the above prepared concentration series in a ratio of 1:1:1 respectively. The spectrophotometer was zeroed using methanol as a blank

solution. The first solution of the series concentration was DPPH with methanol only. The solutions were incubated in a dark place for 30 minute at room temperature before the absorbance readings were recorded at 517 nm.

2.6.5. Percentage of inhibition of DPPH activity

The percentage of antioxidant activity of the plants and the trolox standard were calculated using the following formula:

Percentage of inhibition of DPPH activity (%) = $(A-B)/A \times 100\%$

Where: A, is the optical density of the blank and B, is the optical density of the sample. The antioxidant half maximal inhibitory concentration (IC₅₀) for the plant samples and the standard were calculated using BioDataFit edition 1.02 (data fit for biologist).

2.7. Phytochemical qualitative analysis

The plant aqueous extract were screened for the presence of the phytochemical classes by using the following standard tests [22].

2.7.1. Tests for proteins

Millon's test was performed by mixing 2 ml of Millon's reagent with the entire plant crude extract, appeared white precipitate, which upon gentle heating turned into red color which indicated the presence of protein in the plant. While, Ninhydrin test, was carried out by boiling 2 ml of 0.2% Ninhydrin solution with the entire plant Crude extract, and the appearance of violet color indicated the presence of proteins and amino acids.

2.7.2. Tests for carbohydrates

The presence of carbohydrates was performed using different test. Fehling's solutions test was done by boiling a mixture of Fehling solutions A and B with equal volumes were added to crude plant extract. A red color precipitate indicated the presence of reducing sugars. In addition, Benedict's reagent test was performed by boiling 2 ml of Benedict's reagent with a crude extract a reddish brown color indicated the presence of the carbohydrates. While, Molisch's solution test was carried out by shaking 2 ml of Molisch's solution with crude plant extract then 2ml of concentrated H₂SO₄ was added carefully to the test tube. The appearance of a violet ring at the inter phase of the test tube indicated the presence of carbohydrate. Finally, Iodine test was done by mixing 2 ml of iodine solution with crude plant extract. Purple or dark blue colors prove the presence of the carbohydrate.

2.7.3. Test for phenols and tannins

Two milliliter of 2% FeCl₃ solution was mixed with crude extract. Black or blue-green color indicated the presence of tannins and phenols.

2.7.4. Tests for flavonoids

Flavonoids were tested using Shinoda test. Pieces of magnesium ribbon and concentrated HCl were mixed with crude plant extract, after few minutes pink colored scarlet appeared which indicated the presence of flavonoids. Also Alkaline reagent test was used to check the presence of flavonoids, in which 2 ml of 2% NaOH solution was mixed with plant crude extract, intensive yellow color was formed, which turned into colorless when 2 drops of diluted acid were added to the solution. This result indicated the presence of flavonoids.

2.7.5. Test for saponins

Five milliliters of distilled water were added to crude plant extract in a test tube and the mixture was shaken vigorously. The foam formation indicated the presence of saponins.

2.7.6. Tests for glycosides

Liebermann's test was carried by mixing 2 ml of acetic acid and 2 ml of chloroform together with the entire plant crude extract. The mixture was then cooled and conc. H₂SO₄ was added. The appearance of green color indicated the entity of aglycone steroidal part of glycosides. Also Salkowski's test was done by adding conc. H₂SO₄ (about 2 ml) to the entire

plant crude extract. The appearance of a reddish brown color indicated the presence of steroidal aglycone part of the glycoside.

2.7.7. Keller-kilani test

A mixture of Acetic acid glacial (2 ml) with 2 drops of 2% FeCl₃ solution was added to the plant extract and H₂SO₄ concentrated. A brown ring produced between the layers which indicated the entity of cardiac steroidal glycosides.

2.7.8. Test for phytosteroid

Two milliliter of chloroform and concentrated H₂SO₄ were mixed with the entire plant crude extract. The appearance of a red color in the lower chloroform layer indicated the presence of steroids. Another test was performed by mixing 2 ml of each of acetic acid with conc. H₂SO₄ and crude extract with 2 ml of chloroform. Green color indicated the entity of steroids.

2.7.9. Test for terpenoids

Two milliliter of chloroform were mixed with the plant extract and evaporated on a hot water path then boiled with 2 ml of conc. H₂SO₄. A grey color produced indicated the entity of terpenoids.

2.8. Determination of total phenol content

Total phenol content in the plant was determined using methanolic extracts by spectrophotometric method with some modifications which performed using Folin-Ciocalteu reagent [23]. *R. hybrida* sample: 0.5 ml of methanolic extract of two plant concentrations 1mg/1ml and 0.5 mg/ 1ml was mixed with 2.5 ml of Folin-Ciocalteu reagent (10% in distilled water) and 2.5 ml of 7.5% NaHCO₃. Blank sample: 0.5 ml methanol was mixed with 2.5 ml of reagent and 2.5 ml of 7.5% NaHCO₃. Gallic acid standard solution of 1 mg\1 ml was prepared. This standard solution was used to prepare concentration series to construct the calibration curve by mixing different concentrations of Gallic acid with 2.5 ml of Folin-Ciocalteu reagent (10% in distilled water) and 2.5 ml of 7.5% NaHCO₃. All these samples incubated for 45 minutes at 45°C at thermostat, and the absorbance readings were then determined at λ_{max} 765 nm using a spectrophotometer.

2.9. Determination of flavonoid concentrations in the plant extract

The total flavonoids content was determined from the calibration curve of Rutin and expressed as milligram of Rutin equivalent per gram of extract (mg RU/g extract). Total flavonoid content was determined according to the procedure of (Chang *et al.* 2002) [24], validated by (Nugroho *et al.* 2013) [25] with some modifications using rutin as reference standard. 1mg/1 ml standard solution was prepared, and series concentrations of rutin were then obtained. The sample solution (0.5ml) was added with 1.5 ml methanol, 0.1 ml of 10% AlCl₃, 0.1 ml Potassium acetate 1M and 2.8 ml of distilled water, and then incubated for 30 minutes. Absorbance was measured at λ_{max} 415 nm. Distilled water and AlCl₃ was used as blank. Total flavonoid content was expressed in mg rutin per 1 g of plant extract.

2.10. Data analysis

The antioxidant activity was reported as percentage of inhibition. The inhibition of *R. hybrida* plant and Trolox standard at different concentration were plotted and tabulated and the IC₅₀ for each of them was calculated using the BioDataFit fitting program in which the sigmoidal fitting model was the adapted model.

3. Results and discussion

Ethnopharmacological information and knowledge about herbal products led to many considerable developments in drug discovery and health care systems in the developed and developing countries [26]. With the loss of sudoriferous cultures and rapid industrialization of the planet, most of this information without doubt will disappear. Many of scientific clinical studies supporting that the phytochemical antioxidant compounds in the leaves, fruits and vegetables are the main sufficient factors in reducing the incidence of chronic diseases including heart disease, some types of cancers and other diseases.

The free radical scavenging activity of antioxidants in foods has been substantially investigated and reported in the literature by (Miller *et.al* 2000) [27].

3.1. Phytochemical screening

Qualitative Phytochemical screening tests for aqueous *R. hybrida* extracts showed active phytochemical classes as carbohydrate, reducing sugars, glycosides, flavonoids, terpenoids, phenols and tannin as shown in table 1, all that may be a good reasons to have a potential antioxidant activity due to the high contents of total phenols in which includes terpenoids, flavonoids and tannins[28].

Table1: Qualitative Phytochemical screening tests for the aqueous *R. hybrida* extract.

Phytochemical compound	Test name	Results
Protein & amino acids	Millon test	-
	Ninhydrin test	-
Carbohydrate & reducing sugars	Fehling test	+
	Benedicts test	+
	Molisch test	+
	Iodine test for starch	-
Glycosides	Liebermans test	+
	Salkowski`s test	+
	Keller-kilani test	+
Flavonoid	Alkaline reagent test	+
Saponins	Frothing test	-
Phytosteroids	Libermann Burchard`s test	-
Terpenoid	Copper acetate test	+
Phenolic compounds	Ferric chloride test	+
Tannin	Gelatin test	+
Alkaloids	Wagner`s test	-

3.2. Total flavonoid content

The total flavonoid content for methanolic extract of *R. hybrida* plant presented in Fig. 2, which was calculated from the equation:

$Y = 2.7897x$, it was 53.821 ± 0.42 mg rutin / g of plant extract

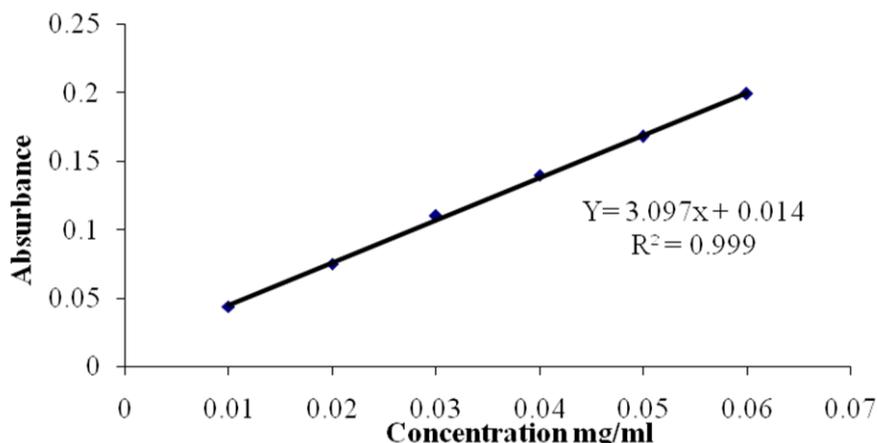


Figure 2: standard calibration curve of Rutin.

3.3. Total phenolic content

Absorbance of standard compound (Gallic acid) at $\lambda_{max} = 765nm$ in *R. hybrida* presented in Fig.3.

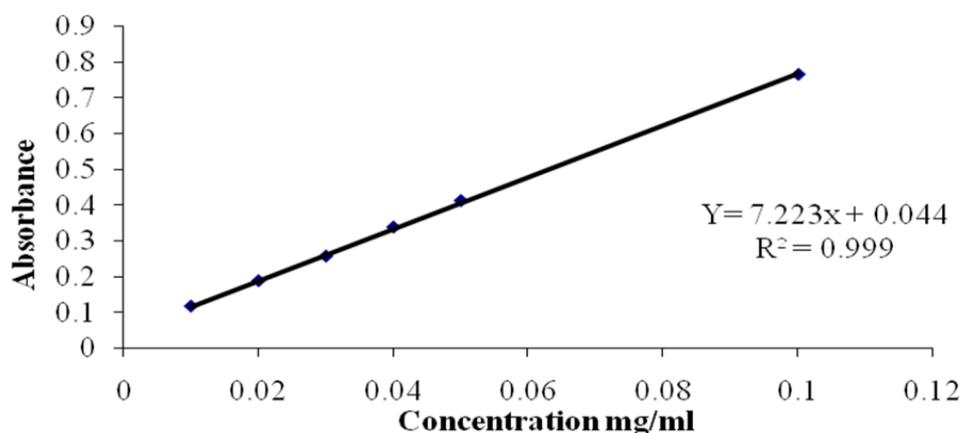


Figure 3: Gallic acid Calibration curve.

From the equation $y = 8.145x$, total phenolic content was 30.448 ± 0.56 mg of GA/g of plant extract.

3.4. Antioxidant activity using Trolox as standard equivalent

The free radical scavenging activity of the methanolic extract of *R. hybrida* has been tested by DPPH radical method using Trolox as a reference standard. The concentration ranged from 1–100 $\mu\text{g/ml}$. The zero inhibition was considered for the solution which contained only DPPH without any plant extract. The results are showed in Table 2, and the table readings are explained in Fig. 4.

Table 2: Percentage inhibition activity for Trolox and *R. hybrida*

Concentration ($\mu\text{g/ml}$)	% inhibition by Trolox $\pm\text{SD}$	<i>R. hybrida</i> Inhibition (%) $\pm\text{SD}$
1	38.6 \pm 1.20	20.2 \pm 1.11
2	49.5 \pm 1.43	25.5 \pm 1.31
3	59.8 \pm 1.66	20.3 \pm 1.16
5	78.8 \pm 1.76	27.8 \pm 1.17
7	88 \pm 1.86	29.4 \pm 1.06
10	97.2 \pm 1.45	30.3 \pm 1.09
20	97.2 \pm 1.36	42.1 \pm 1.11
30	97.5 \pm 1.78	43.2 \pm 1.53
40	97.5 \pm 1.84	43.4 \pm 1.21
50	97.2 \pm 1.35	51.2 \pm 1.26
80	97.2 \pm 1.35	68 \pm 1.78
100	97.2 \pm 1.62	78 \pm 1.98

Free radical scavenging capacities (IC_{50}) of *R. hybrida* extract measured in DPPH. The following curve (Fig.4) show the inhibition activity for both Trolox and plant.

Then to compare the antioxidant activity of the plant IC_{50} should be calculated for both Trolox and plant to compare between them, IC_{50} of plant was 31.405 ± 0.21 $\mu\text{g/ml}$ while for Trolox standard reference was 2.108 ± 0.44 $\mu\text{g/ml}$.

Regarding to these results, there is a relationship between total phenols contents and antioxidant activity in *R. hybrida* plant. The total phenolic content investigated in this study for *R. hybrida* was 30.448 ± 0.56 mg of GA/g of dry plant powder as well as the free radical scavenging capacity (IC_{50}) was 31.405 ± 0.21 $\mu\text{g/ml}$, meanwhile the total flavonoids contents was 53.821 ± 0.42 mg rutin/g of plant extract, that means that the *R. hybrida* plant may has an antioxidant activity due to the presence of phenolic compounds, also these results indicated that not

all of the flavonoids in this plant react with DPPH which give them a negative correlation with total phenols and antioxidant activity.

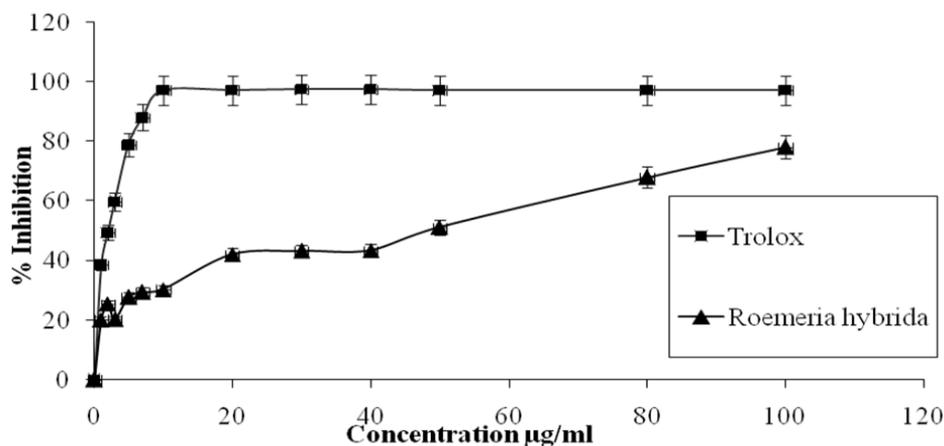


Figure 4: Inhibition activity of Trolox standard and *R. hybrida* extract.

However, these results indicate that when the plant materials were included in the statistical analysis, there was a positive and highly significant ($p < 0.005$) relationship between total phenols contents and antioxidant activity. In Palestinian flora growing wildly two species of Roemeria plants, *Roemeria refracta* and *Roemeria hybrida*, *R. refracta* showed less potential antioxidant activity ($54.3\mu\text{g/ml}$) than *R. hybrida* according to the study conducted by Souri *et al*, in 2010 on this species [29].

All these results indicated that *R. hybrida* is the best source for antioxidant supplement from all Roemeria species growing in Palestine and it has approved its folk uses in treatment of diabetes mellitus and other diseases associated with free radical stress.

4. Conclusion

The phytochemical screening showed that the *R. hybrida* plant extract contain a mixture of phytochemicals and the quantitative tests indicated that the methanolic plant extract has promising contents of flavonoids and phenols and has potent antioxidant activity, all that may proved its folk uses in treatments of various diseases associated with oxidative stress in the body. In addition to that *R. hybrida* can be an excellent choice for further clinical studies and to be utilized in the modern medicine for manufacturing of medicaments for treatment of various diseases.

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