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Research Article

Preliminary Phytochemical and Biological Screening of *Cyclamen coum* a Member of Palestinian Flora

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ABSTRACT

Background: Since ancient times, the treatment of various diseases has relied on medicinal plants. The replacement of chemically synthesized drugs with natural medications gave the later advantageous due to their safety for humans. This study aimed to screen and to evaluate preliminarily phytoconstituents, total contents of flavonoids, tannins, and phenols also to evaluate the antibacterial and antioxidant activities of *Cyclamen coum* aerial parts methanol extract.

Methods: Screening of phytoconstituents, total flavonoids, tannins and phenols contents, as well as antioxidant properties, were investigated for the *Cyclamen coum* aerial parts by using standard phytochemical and analytical methods. Evaluation of antibacterial activity of the plant methanolic extract was performed by using broth microdilution method. American Type Culture Collections of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and Methicillin-Resistant *Staphylococcus aureus* (MRSA) were utilized to evaluate antibacterial activity.

Results: Preliminary phytochemical qualitative analysis of the plant methanolic extract showed that the plant aerial parts contained amino acids, reducing sugar, carbohydrate, tannin, flavonoid, phenol, saponin, cardiac glycoside, and steroids. Total flavonoids, phenols, and tannins in plant methanolic extract were 60.88 mg QUE/g, 32.7 mg GAE/g and 11.7 mg CAE/g, respectively. Meanwhile, the antioxidant activity was estimated to be 31 µg/mL and has very weak antibacterial activity on the studied pathogens.

Conclusion: In the present study, evaluation of total phenolic, tannins and flavonoid content from the aerial parts of *C. coum* can be an antioxidant naturally potent source. The results of *in-vitro* antibacterial studies showed that the studied plant has very weak antibacterial activity. In addition, the presence of antioxidant compounds in *C. coum* makes it a good candidate for manufacturing dietary supplements and food preservatives.

Introduction

Herbal medicines were used for therapeutic purposes since the beginning of human history and have a long usage outside conventional medicine. When the use of herbs started, it was associated with the pharmacological treatment of diseases.[1] In fact, a great proportion of the population in the developing world relies on traditional herbal healers who use a large number of herbs in order to meet health care needs in these countries, and many conventional drugs still in use in the pharmaceutical industry are derived from or extracted from herbs.[2,3] In fact, many researchers have shown an increasing interest in herbs in developed and developing countries and in many pharmaceutical, biological, phytochemical and medical fields and thousands of experiments are conducted yearly in vitro and in-vivo to investigate the potential of new drugs derived from plants.[4,5]

Cyclamen is the plant genus with about 23 species, which widely distributed mainly in the coastal areas of the Mediterranean Sea and Black Sea regions especially in Turkey, Georgia, Bulgaria, Syria, Lebanon and Palestine.[6]

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Cyclamen coum Mill. belongs to the Primulaceae family and has common names such as Eastern cyclamen, Round-leaved cyclamen, Zaamatot, which can be briefly described as a perennial herbaceous plant, which reaches about 10 cm in height, with rounded leaves sometimes marbled with silver spots on the upper surface. The 2 cm in width deep pink flowers have purple blotch at the base of each lobe. It grows in shady places in coniferous and broad-leafed woodland and scrubs, sometimes growing amongst rocks and tree roots.[6,7]

The whole *C. coum* plant contains sterols,[8] piperidine alkaloid 2-β-D- glycopyranosyl-2-undecil-3,5-dihydroxy-6-carboxypiperidine,[9] and saponins as cyclaminorin, coumoside A, coumoside B, cyclacoumin, isocyclamin, deglucocyclamin and mirabilin lactone.[10-13]

In folk medicine, the *C. coum* has been used for treatment of hemorrhages, ecchymosis, inflammations, hemorrhoids and cancer.[14,15]

An evidence-based study conducted by Yildiz *et al.* proved that *C. coum* crude extract induced moderate cytotoxicity and apoptosis in NSCLC H1299 and HeLa cancer cell lines and the degree of apoptosis and cytotoxicity on cervical cancer cell line HeLa is stronger than on NSCLC H1299 cell line.[15]

Another evidence-based study performed by Shafiei *et al*. showed n-butanol *C. coum* extract had antibacterial activity against planktonic cells of *P. aeruginosa* strains due to the presence of saponin content. Moreover, this study proved that this extract in combination with ciprofloxacin showed to be more effective against *P. aeruginosa* bio-films than ciprofloxacin used alone.[16]

However the problem of antibacterial resistance has increased worldwide, and the need to solve this problem and develop new drugs, natural or synthetic, has become more and more urgent (18).

In addition, many of the recently conducted studies in medical and pharmaceutical fields showed a huge number of diseases associated with free radicals. The risk of diseases due to oxidative stress is compounded by pollution, unhealthy lifestyle, cigarette smoking, exposure to chemicals, drugs, illness, stress and other factors.[17-19] On the other hand, there is a shortage of literature about the antibacterial and antioxidant activity of *C. coum*. Accordingly, this research is aimed to conduct the phytoconstituents, total phenols, flavonoids and tannins contents as well as antioxidant and antibacterial activities of the local growing plant.

Materials and Methods

Chemical reagents and solvents

The chemical reagents and solvents that were used for the estimations of the antioxidant potential included: methanol (Lobachemie, India), n-hexane, Trolox ((s)-(-)-6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) (Sigma-Aldrich, Denmark), (DPPH) 2,2-Diphenyl-1-picrylhydrazyl (Sigma-Aldrich, Germany). For phytochemical screening, the utilized reagents included: Millon's reagent, Ninhydrin solution (Alfa Agar, England), Benedict's reagent, Molish's reagent, H_2SO_4 and iodine solution (Alfa-Aesar, England), NaOH, chloroform, HCl (Sigma-Aldrich, Germany), magnesium ribbon, acetic acid, $FeCl_3$ (Riedeldehan, Germany).

Folin-Ciocalteu's reagent (Sigma-Aldrich, Germany) was utilized for the evaluation of the total phenol contents. The reagents, utilized for evaluation of total flavonoids contents, included: quercetin, (Sigma-Aldrich, Denmark), $AlCl_3$ and potassium acetate (Sigma-Aldrich, Germany), while catechin and vanillin (Sigma-Aldrich, Germany), compounds were used to evaluate total tannin content.

Mueller-Hinton (MH) broth and dimethyl sulfoxide (DMSO) (Riedeldehan, Germany) were used for antibacterial screening experiments.

Instrumentation

Rotary evaporator (Heidolph VV2000, Germany), freeze dryer (Mill rock technology, model BT85, China), grinder (Moulinex model, China), balance (Radwag, AS 220/c/2,Poland), filter papers (Machrery-Nagel, MN 617 and Whatman no.1, USA), micropipettes (Finnpipette, Finland), syringe filter with pore size 0.45 *µm* (Microlab, China), micro broth plate (Greiner bio-one, North America) and incubator (Nuve, Turkey).

Collection and preparation of the plant materials

The aerial parts of *C. coum* plant were collected in April 2015 from the Jerusalem Mountains in Palestine. The plant was identified by the pharmacognosist Dr. Nidal Jaradat and the voucher specimens were deposited in the Pharmacognosy laboratory, Faculty of Medicine and Health Sciences at An-Najah National University and the voucher specimen code was Pharm-PCT-776.

The collected aerial parts of *C. coum* plant were washed with distilled water and later completely dried in shade at room temperature; the dried parts were grounded into a fine powder using a mechanical blender and stored in tightly sealed special containers until use.

Preparation of the plant methanolic extract

One liter of methanol (99%) was used to soak about 10 gram of the ground plant then at room temperature it was put in a shaker device at 100 rpm for 72 hours and stored in a refrigerator for 4 days. The extract was filtered using filter papers and concentrated on a rotary evaporator under vacuum. Finally, the crude extract was stored at 4ºC for further use.[20,21]

Antioxidant activity

A stock solution of a concentration of 1 mg/mL in methanol was initially prepared for plant extract and Trolox standard. Stock solutions were used to prepare working solutions with the following concentrations (1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80, 100 µg/mL) by using serial dilution in methanol.[22]

A solution of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was freshly prepared at a concentration of 0.002% w/v. Then, it was mixed with methanol along with each of the working concentrations in a ratio of 1:1:1. Methanol was used as a blank solution for zeroed the spectrophotometer. The first solution of the series concentrations was DPPH with methanol only. The solutions were incubated at room temperature in a dark cabinet for about 30 minutes. Then, their optical densities were determined by using the spectrophotometer at a wavelength of 517 nm.

The percentage of antioxidant activity of plant extract and the Trolox standard was calculated using the following formula:

$$DPPH\ inhibition\ activity(\%) = \frac{(B-S)}{B} \times 100\% \quad \text{Eq. (1)}$$

Where B is the blank optical density and S is the sample optical density.

The antioxidant half maximal inhibitory concentration (IC_{50}) for the plant extract as well as the standard deviation were both calculated using BioDataFit edition 1.02 (data fit for biologist). The antioxidant activity was reported as the percentage of inhibition. The inhibition of Trolox standard and the plant extract, at different concentrations, were plotted and tabulated. Then, the BioDataFit program was used to calculate the IC_{50}.

Qualitative phytochemical analysis

The initial preliminary phytochemical screening of primary and secondary metabolic compounds such as glycosides, proteins, starch, phenols, saponin glycosides, flavonoids, steroids, tannins and volatile oils were conducted according to standard phytochemical methods which described by Evans[23] and Harborne.[24]

Determination of total phenol content in the methanolic extract

Total phenolic content in *C. coum* methanolic extract was determined using the spectrophotometric method with some modifications.[25] One mg/mL aqueous solution of methanolic *C. coum* extract was prepared to determine the total phenolic content. The reaction mixture was prepared by mixing 0.5 mL of plant extract solution, 2.5 mL of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 mL of 7.5% of the $NaHCO_3$ aqueous solution. The samples were later incubated in a thermostat at 45ºC for 45 minutes. The wavelength 765 nm was fixed, to determine the absorbance on the spectrophotometer. The absorbance was calculated as the mean value of each analyzed sample after it was prepared in triplicate. The standard solution of Gallic acid and the calibration line was construed using the same procedure. The Gallic acid equivalent concentration was expressed in terms of (mg of GAE/g of extract) based on the measured absorbance.

Determination of total flavonoids contents in the methanolic extract

Quercetin calibration curve was used to determine the total flavonoid content, and it was expressed as milligram of Quercetin equivalent per gram of extract (mg QUE/g extract). According to the procedure of Chang *et al*., the determination of the total flavonoid content was carried out and the procedure was validated by Nugroho with some modifications.[26,27] Quercetin (10 mg) was dissolved in 10 mL of distilled water and was diluted to 100 mL. Subsequently, the stock solution was diluted to provide a series of concentrations (10, 30, 40, 50, 70, 100 µg/mL); from each solution, (1 mL) was mixed with 0.3 mL of 10% $AlCl_3$, 0.3 mL of sodium nitrite, 1 mL of 2M NaOH and 1 mL of distilled water. The samples were incubated at room temperature for 30 minutes. The absorbance was determined at wavelength 510 nm using a spectrophotometer. Total flavonoid in extracts was expressed in terms of Quercetin Equivalents (mg of QUE/g of *C. coum* extract).

Determination of total Tannin content in the plant methanolic extract

The method of Sun *et al.*[28] was used to determine the total tannin content in the plant methanolic extract. To 0.5 mL of each diluted Catechin solutions which concentrations were (0.1, 0.4, 0.5, 0.7, 1mg/mL), 3 mL of 4% vanillin solution in methanol and 1.5 mL of concentrated HCl were added. The mixture was left to stand for 15 minutes and absorption was measured at 500 nm against methanol as a blank. The value of total tannins is expressed as mg (+)-catechin/g of plant extract. The same procedure was repeated for the plant methanol extract. All samples were analyzed in triplicate

Statistical analyses

The results were expressed as a mean ± standard error for each sample after the determinations were conducted in triplicate. Statistical analyses were performed using one-way ANOVA followed by Dunnet's test with p-value < 0.05 as a limit of significance.[29]

Measuring the minimum inhibitory concentration (MIC)

Minimum inhibitory concentration was determined for the methanol plant extract (dissolved in 10%

DMSO) by broth microdilution technique for all tested microorganisms according to.[30,31] The antibacterial activities of the extracted plant were examined against the growth of four reference bacterial strains obtained from the American Type Culture Collection (ATCC); *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853) as well as against the growth of a diagnostically-confirmed Methicillin-Resistant *Staphylococcus aureus* (MRSA) isolate. The wells were filled with 100 µL Mueller-Hinton (MH) broth solution, 100 µL of 50 mg/mL of methanolic extract of *C. coum* was added to the first well of micro-dilution tray and after mixing, 100 µL was transferred to the second well, then to the third and so on; until eleventh well, where 100 µL were discharged after mixing. Positive control of bacterial growth was well number 12. To detect any antibacterial activity of DMSO, 10% DMSO was serially diluted with broth in 2 separate rows. Finally, 1 µL of bacterial diluted suspension (5×10^7 CFU/mL) was added to all wells except well number 11, which was considered the negative control of bacterial broth. Each plant extract was made in duplicate. The inoculated microdilution plate was incubated at 35ºC for 24 hours. Well with the lowest concentration of plant extract in each row that did not show visible bacterial growth was considered as MIC well.[30,31]

Results

Phytochemical screening

Phytochemical qualitative analysis of *C. coum* extract showed that the aerial plant parts contained amino acids, protein, reducing sugar, carbohydrate, tannin, flavonoid, phenol, saponin, cardiac glycoside, and steroid (Table 1).

Table 1. Phytochemical tests for the methanolic extract of *C. coum*.

Phytochemical compound	Methanolic extract
Amino acids and protein	+++
Reducing sugar	++
Carbohydrate	+++
Tannin	++
Flavonoid	++
Phenol	+++
Saponin	+
Cardiac glycoside	++
Steroid	++
Alkaloids	-

Where; (-) means no content, (+) low content, (++) mild content, and (+++) high content

Total flavonoid content

Total flavonoid content in the methanolic plant extract was determined. The results of total flavonoid content were expressed by mg/mL quercetin equivalent using the standard curve equation:

$$Y = 0.003X - 0.021, R^2 = 0.9935 \quad \text{Eq. (2)}$$

Where Y is Absorbance at 510 nm and X is flavonoid concentration in the plant extract.

Total flavonoid concentration content of the plant extract was 60.88 mg QUE/g of plant methanolic extract.

Total phenolic content

Total phenolic content in the extract was estimated using Folin Ciocalteu's method and the standard compound (Gallic acid).

The results of total phenolic content were expressed by mg/g gallic acid equivalent using the standard curve equation

$$Y = 7.277x + 0.044, R^2 = 0.98893 \quad \text{Eq. (3)}$$

Where Y is Absorbance at 765 nm and X is total phenol in the plant extract.

The total phenolic content of the plant extract was 32.7 mg GAE/g of extract.

Total tannins content determination in methanolic extract

The tannin content was examined in plant extract using catechin and vanillin reagents is expressed in terms of catechin equivalent (the standard curve equation: y=0.955x, R^2= 0.999x). The values obtained for the concentrations of tannin contents are expressed as mg (+)-catechin/g plant extract. From the calibration curve the results showed that the total Tannin content was 11.7 mg CAE/g of plant methanolic extract.

Antioxidant capacity

The free radical scavenging activity of the *C. coum* methanol extract has been determined by DPPH radical method using Trolox as a reference standard. The concentration ranged from 1-100 µg/mL. The zero inhibition was considered for the solution which contained only DPPH without any plant extract. The half maximal inhibitory concentration (IC_{50}) was 31 µg/mL for *C. coum* and 2 µg/mL for Trolox.

The results of total flavonoid, phenol and tannins contents as well as the IC_{50}'s of the plant methanolic extract and Trolox are presented in Table 2.

The results in Figure 1 showed that at 30 mcg/ml for the plant extract, the forward reaction with free radical reaches maximum extent and after this concentration the reverse reaction will begin and free radical will form again.

Antibacterial activity

Methanolic extract showed antibacterial activity with MIC value ranging between 6.25-12.5 mg/mL against different bacterial strains including *S. aureus*, *E. coli*, and *P. aeruginosa* as well as MRSA as shown in Table 3.

Table 2. Antioxidant, total flavonoid, phenolic and tannins results.

Phytochemical tests	The aerial parts of *C. coum* methanolic extract
Total flavonoid contents	60.88±1.3 mg QUE/g plant extract
Total phenolic contents	32.7±1.7mg GAE/g plant extract
Total tannins contents	11.7±0.91 mg CAE/g plant extract
Antioxidant activity (IC_{50})	31±1.9 µg/mL plant extract
Trolox (IC_{50})	2 µg/mL

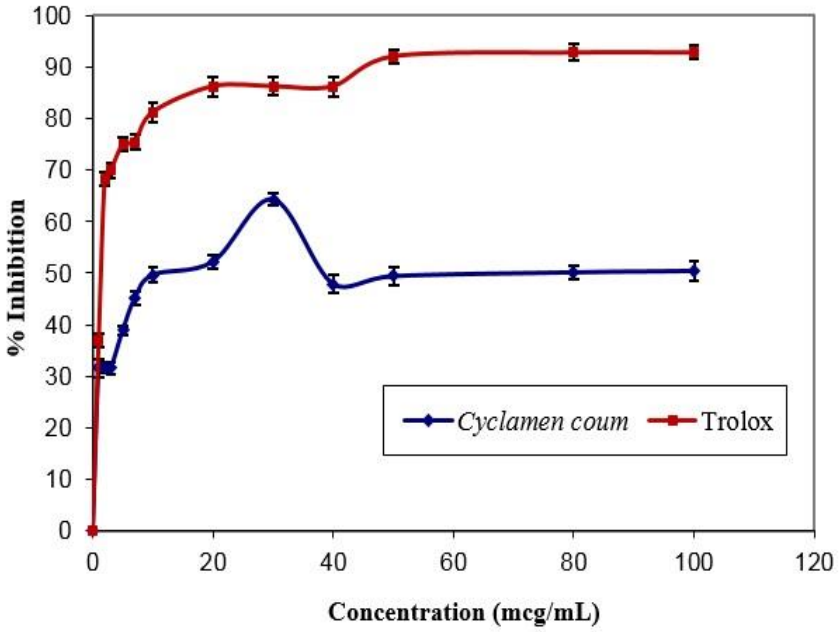

Figure 1. Inhibition activity of Trolox standard and *C. Coum.*

Table 3. MIC of *C. coum* methanolic extract (mg/mL).

Microorganism	MIC mg/mL
Staphylococcus aureus (ATCC 25923)	12.5
Escherichia coli (ATCC 25922)	12.5
Pseudomonas aeruginosa (ATCC 27853)	6.25
Staphylococcus aureus (MRSA Positive)	12.5

The growth of the studied bacterial reference strains was not inhibited by 5% DMSO (10% DMSO was diluted 1:2 in the first well in each row).

Discussion

Recently, plants utilization in medicine is becoming more valuable due to improvements in analysis and quality control, along with advances in clinical research which showed their values in the prevention and treatment of various diseases. It is realized in the last decades the importance of herbal medicine to improve health care systems generally.[32] The Folin-Ciocalteu's method gives a crude assessment of the total phenol present in the plant extract. It is not specific to polyphenols, but many interfering compounds may react with the reagent, giving elevated apparent phenolic concentrations.[33] In comparison with *C. persicum* the most common species of *Cyclamen* genus, the studied *C. coum* showed higher contents of phenols than *C. persicum*, which was evaluated by Tawaha *et al.* 2007 (17.0 ± 0.6 mg GAE/g extract) while *C. coum* total phenols content was 32.7 mg GAE/g of plant methanolic extract.

Flavonoids occurring in the plants can be divided into six major subgroups: flavonols, chalcones, flavan-3-ols, flavones, proanthocyanins and anthocyanins.[34] They are polyphenolic antioxidants, which possess key pharmacological activities, including antibacterial, antioxidant and anticarcinogenic properties.[35] The aerial parts of *C. coum* plant showed high total flavonoid content which was 60.88 mg QUE/g of plant methanolic extract while the total tannin content was 11.7 mg CAE/g of plant methanolic extract, which is considered a moderate amount in comparison with other plants tannin contents.

Interestingly, *C. coum* leaves, which showed good

antioxidant activity in this study are being consumed by local people in Palestine and Jordan.[36] Comparison of the antioxidant activity for *C. coum* species, showed better antioxidant activity than wild and cultivated *C. persicum* when it's antioxidant potential was assessed by Jaradat *et al*., and their IC_{50} was 38.2 µg/mL and 39.8 µg/mL respectively while *C. coum* IC_{50} was 31µg/mL.[20]

Antibacterial activity of *C. coum* was evaluated by using ATCC strains of *S. aureus*, *E. coli* and *P. aeruginosa* as well as MRSA and the results showed that this plant had weak antibacterial activity. The best and potential MIC was 6.25 mg/mL against *P. aeruginosa*, this bacteria may cause respiratory system, bones, joints, urinary tract, soft tissue and gastrointestinal infections also may cause dermatitis, bacteremia and a variety of systemic infections, particularly in patients with severe burns, cancer and AIDS.[37]

Therefore, this particular finding should be extensively studied in order to use the *C. coum* in the protection of many diseases due to its antioxidant potentials.

Conclusion

The *C. coum* showed very weak activity against the studied bacterial strains. It contains respective amounts of phenols and flavonoids as well as has mild antioxidant activity. The *C. Coum* may be used as a good candidate for the manufacturing of new food preservatives and dietary supplements due to its antioxidant potentials. Further investigations are required to extend its active phytoconstituents as well as its pharmacological and clinical applications.

Conflict of interests

The authors claim that there is no conflict of interest.

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