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Research article

Quantitative determination of phytochemical constituents of *Viola lutea* by HPLC

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Abstract

Phytochemical screening revealed the presence of various bioactive secondary metabolites as flavonoids, phenolics, saponins, glycosides, cardiac glycosides, tannins and alkaloids. Anthraquinones and sterols were not detected in the plant. The percentage of inorganic matter, organic matter, crude fibers content, total carbohydrates, total nitrogen, total proteins, total lipids, total tannins, total saponins, total alkaloids, total flavonoids, total phenolic of the plant were determined in this study. The flavonoid and phenolic contents of *Viola lutea* were analyzed by High performance liquid chromatography (HPLC). The quantification of each compound was done according to the peak area measurements which were reported in calibration curves of the corresponding standards. The result revealed that *Viola lutea* consists of flavonoid of, myricetin, luteolin and apigenin 8.44, 23.1 and 15.4 mg/ml, in corresponding to RT values 8.14, 9.23 and 11.78 min, respectively. On the other hand; protocatechuic acid, benzoic acid and Syriniginic acid as phenolic compounds appeared with concentrations 5.66, 8.65, and 12.5 mg/ml in corresponding to RT values 8.07, 9.64 and 10.71nm, respectively.

Introduction

The use of plants in the pharmacological treatment of disease began long ago. Centuries ago, Chinese, Japanese, and Indian used herbs in disease treatment as traditional medicine [1, 2]. Plants have medicinal importance especially as antimicrobial activity and those are rich in their phytochemical contents whether alkaloids, flavonoids, etc. which encourages the researchers to investigate more uncommon plants for their medicinal importance [3]. The plant genus *Viola*, often known as violets or pansies. *Viola* is the largest genus in the family Violaceae, containing between 525 and 600 species. *Viola lutea,* also known as the mountain pansy. *Viola lutea* grows to a height of around 20 cm (8 in). Its flowers are 20–35 mm (0.8–1.4 in) in diameter, and are typically yellow, although some individuals may have blue, purple or blotched flowers instead [4]. The medicinal usage of genus *Viola* is commonly used as remedy for coughs, sore throat, hoarseness and tonsillitis. It is valued as an expectorant, antioxidant, diaphoretic, antibacterial, antipyretic, diuretic and as a laxative. The pharmacological studies revealed the role of *Viola* in some Unani drugs for treatment of common cold, asthma, antimicrobial, and cough associated diseases. It is rich in many phytoconstituents such as, saponins, salicylates, alkaloids, flavonoids, saponins, tannins, phenolics, coumarins, phenolic glycosides, gaultherin, violutoside, saponins, flavonoids, and odoratine [5].

Materials and methods

Plant material

The plant specimens of *Viola lutea* had been provided by Agriculture Research Center (ARC), Dokki, Giza-Egypt.

Sample preparation
The powdered plant material aerial parts (100 grams) from *Viola lutea* was extracted with minimum amount of 70% ethanol, and purified according to standard procedures reported by [6,7]. The slurry was allowed to stand for 24 h with occasional stirring, and then filtered off. The residue was repeatedly extracted with an excess volume of 70% ethanol. Combined filtrates were evaporated under reduced pressure using rotavapour apparatus at 60ºC for 15 minutes until a minimum amount of solvent remained.

Preliminary qualitative phytochemical screening
The crude ethanolic 70% extracts of *Viola lutea* plant was subjected to preliminary qualitative phytochemical screening for the presence of bioactive constituents using standard phytochemical techniques as described by [8-11].

Test for carbohydrates and/or glycosides
Liebermann's Test: We added 2.0 ml of acetic acid and 2 ml of chloroform with whole aqueous plant crude extract. The mixture was then cooled and we added H_2SO_4concentrated. Green color showed the entity of aglycone, steroidal part of glycosides.

Keller-Kiliani Test: A solution of glacial acetic acid (4.0 ml) with 1 drop of 2.0% $FeCl_3$ mixture was mixed with the 10 ml aqueous plant extract and 1 ml concentrated H_2SO_4. A brown ring formed between the layers which showed the entity of cardiac steroidal glycosides.

Salkowski's Test: We added 2 ml concentrated H_2SO_4 to the whole aqueous plant crude extract. A reddish brown color formed which indicated the presence of steroidal aglycone part of the glycoside.

Test for flavonoids
Shinoda Test: Pieces of magnesium ribbon and Hcl concentrated were mixed with aqueous crude plant extarct after few minutes and pink color showed the presence of flavonoid.

Alkaline Reagent Test: 2 ml of 2.0% NaOH mixture was mixed with aqueous plant crude extract; concentrated yellow color was produced, which became colorless when we added 2drops of diluted acid to mixture. This result showed the presence of flavonoids.

Test for saponins
5.0 ml of distilled water was mixed with aqueous crude plant extract in a test tube and it was mixed vigorously. The frothing was mixed with few drops of olive oil and mixed vigorously and the foam appearance showed the presence of saponins.

Test for tannins
10 ml of bromine water was added to the 0.5 g aqueous extract. Decoloration of bromine water showed the presence of tannins.

Test for terpenoids
2.0 ml of chloroform was added with the 5 ml aqueous plant extract and evaporated on the water path and then boiled with 3 ml of H_2SO_4concentrated. A grey color formed which showed the entity of terpenoids.

Test for steroids
2 ml of chloroform and concentrated H_2SO_4 were added with the 5 ml aqueous plant crude extract. In the lower chloroform layer red color appeared that indicated the presence of steroids.

Test for Anthraquinones
10 ml of benzene was added in 6 g of the Ephedra powder sample in a conical flask and soaked for 10 minutes and then filtered. Further 10 ml of10% ammonia solution was added to the filtrate and shaken vigorously for 30 seconds and pink, violet, or red color indicated the presence of anthraquinones in the ammonia phase.

Determination of certain pharmacopeial constants of plant materials including inorganic (ash) and organic matter [12], acid-soluble and acid-insoluble ash, water-soluble and water- insoluble ash [13] and crude fibers [14].

Investigation of metabolic products including determination of total carbohydrates, soluble and insoluble carbohydrates [15], Total nitrogen and protein content in *Viola lutea* were determined using Kjeldahl method [16]**.** Total lipids content, acid value [17], ester value [18], saponification value [19], iodine value [20].

Investigation of total active materials
Estimation of total tannins using gravimetric method (Copper acetate method) according to [21], estimation of total saponins according to [22], estimation of total alkaloids (Gravimetric method) were carried according to the method described by [23] and the amount of total phenolics and flavonoids were determined according to methods of [24, 25].

Qualitative and quantitative determination of phenolics and flavonoids of *Viola lutea* plant using HPLC was carried out according to the method described by [26]. High performance liquid chromatography (HPLC)technique using HPLC Agilent 1100 series equipped with Quaternary pump, set at flow 1ml/min. Autosampler, degasser, column compartment set at 35ºC and variable wave length detector set at 330 nm for flavonoid compounds and 280 for phenolic compounds .Column: Hypersil ODS

5µm, 250x4 mm was used. This work was carried out in the Food Technology Research Institute, Agric. Res. Center, Giza, Egypt.

Results and discussion

Phytochemical evaluation was performed for qualitative and quantitative detection of various chemical constituents which aid in tracing the presence of active entity that elicit a major biological response of the plant. In the present work, the preliminary phytochemical screening of *Viola lutea* plant proved the presence of various bioactive secondary metabolites as flavonoids, phenolics, saponins, glycosides, cardiac glycosides, tannins and alkaloids. Anthraquinones and sterols were not detected in the plant as represented in table 1.

Kayani *et al.* recorded that, these secondary metabolites are chemicals produced by means of secondary reactions resulting from primary carbohydrates, amino acids and lipids [27]. These phytochemical constituents are of physiological importance and possess hypolipidemic, anti-tumor or stimulating properties which can reduce the risks of cardiovascular disease and cancer [28].

Certain pharmacopeial constants of *Viola lutea* including inorganic (ash) and organic matter, acid-soluble and acid-insoluble ash, water-soluble and water- insoluble ash and crude fibers are summarized in table 2. Higher ash content indicates that, the total inorganic mineral is high [29]. Also, Smith, stated that, the high content of ash is useful in assessing the minerals present in the sample [30].

It was observed from the obtained data that, the percentage of crude fibers in plant reached to17.17 ±0.15. Fibers used as prebiotic, where it has the ability to promote bacteria fermentation in colon. Dietary fibers play an important role in human health, which consists mainly of cellulose, hemicelluloses and lignin, which exert different physiological effects on human health [31]. Food fiber promotes absorption of trace elements in the gut; reduce absorption of cholesterol and lower blood glucose in diabetic patients [32].

The metabolic products of *Viola lutea* including determination of total carbohydrates, soluble and insoluble carbohydrates, Total nitrogen, protein content, total lipids content, acid value, ester value, saponification value and iodine value are summarized in table 3.

The percentage of total lipids content in plant may be due to an increase in the metabolic rate, which leads to increase in carbohydrate concentrations that convert to lipid by oxidation reaction [33].

It was also, observed that, the percentage of total nitrogen and total protein reached to (1.76±0.13and 11.04±0.33), respectively in the plant. Nitrogen is a universally occurring element in all living beings and major component of protein, therefore the concentration of protein is closely linked to the concentration of nitrogen in the plant.

The obtained results in table 4 declared that, total active materials in *Viola lutea* plant like flavonoids reached to 248.33±1.13mg/g rutin and total phenolics reached to 315.33±1.07mg/g gallic acid, flavonoids and other plant phenolics are reported to have multiple biological activities in addition to their antioxidants or free radical terminators activity [34]. Therefore, it is worth, while to determine their total amount in the plant chosen for the study.

Table 1. Phytochemical constituents in *Viola lutea* plant.

Bioactive constituents	Observation
Flavonoids	+
Phenolics	+
Saponins	+
Glycosides and/or carbohydrates	+
Cardiac glycosides	+
Tannins	+
Alkaloids	+
Sterols and/or terpenes	-
Anthraquinones	-

Table 2. Certain pharmacopeial constants of *Viola lutea* plant.

Item %	Mean ± SE
Total ash	19.23±0.26
Organic matter	80.43±0.17
Acid soluble ash	11.40±0.11
Acid insoluble ash	7.83±0.27
Water soluble ash	12.06±0.19
Water insoluble ash	6.87±0.35
Crude fibers	17.17±0.15

Data are presented as mean ± SE for 3 replicates. SE: Standard error.

Table 3. The metabolic products of *Viola lutea.*

Item %	Mean ± SE
Total carbohydrates	31.63±0.36
Soluble carbohydrates	19.20±0.22
Insoluble carbohydrates	12.43±0.25
Total nitrogen	1.76±0.13
Total protein content	11.04±0.33
Total lipids content	1.27±0.13
Acid value	16.17±0.29
Ester value	97.20±0.42
Saponification value	113.37±0.49
Iodine value	82.00±0.40

Data are presented as mean ± SE for 3 replicates. SE: Standard error.

Table 4. The total active materials in *Viola lutea* plant.

Item	Mean ± SE
Total Flavonoids (mg/g rutin)	248.33±1.13
Total phenolics (mg/g gallic acid)	315.33±1.07
Total saponins (%)	1.13±0.08
Total tannins (%)	1.23±0.08
Total alkaloids (%)	1.7±0.11

Data are presented as mean ± SE for 3 replicates. SE: Standard error.

In the course of the present study, some effective flavonoid and phenolic compounds in *Viola lutea* were identified by HPLC. The result revealed that *Viola lutea* consists of flavonoid of myricetin, luteolin, and apigenin (8.44, 23.1, and 15.4 mg/ml), with retention time (8.14, 9.23 and 11.78 min), respectively (Table 5 & Figure 1) and phenolic of protocatechuic acid, benzoic acid and Syriniginic acid (5.66, 8.65, and 12.5 mg/ml) with retention time (8.07, 9.64 and 10.71 min), respectively (Table 6 & Figure 2).

Table 5. The flavonoid contents of *Viola lutea* by HPLC.

Peak	RT (min)	Compound	Concentration (mg/ml)
1	8.14	Myricetin	8.44
2	9.23	Luteolin	23.1
3	11.78	apigenin	15.4

Table 6. The phenolic contents of *Viola lutea* by HPLC.

Peak	RT (min)	Compound	Concentration (mg/ml)
1	8.07	Protocatechaulic acid	5.66
2	9.64	Benzoic acid	8.65
3	10.71	Syriniginic acid	12.5

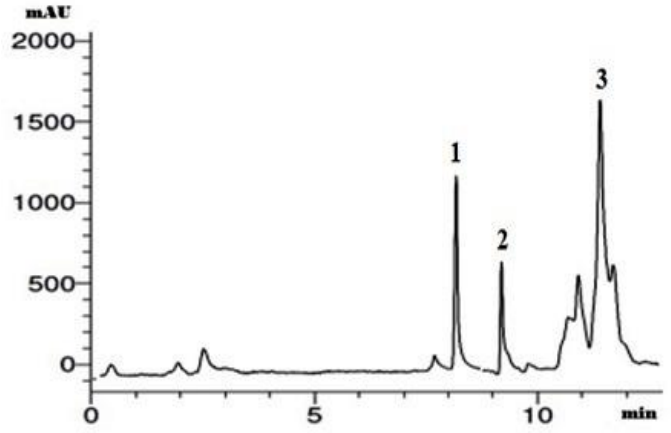

Figure 1. The flavonoid contents of *Viola lutea* by HPLC.

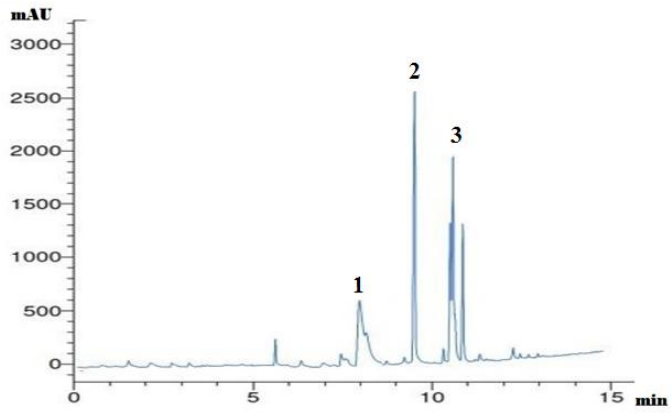

Figure 2. The phenolic contents of *Viola lutea* by HPLC.

Conclusion

Conclusively, *Viola lutea* is rich in its phytochemical composition which encourages the researchers to evaluate its pharmacological activity especially due to its flavonoids, phenolics and alkaloids. HPLC succeeded to determine both flavonoids and phenolics of *Viola lutea* according to the authentic references used in detection process.

Conflicts of Interest

The authors declare no conflict of interest.

Author contributions

All the authors have contributed equally in designing, drafting the manuscript as per the journal submission format. All authors read and approved the final manuscript.

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