Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(3): 3219-3223
Received: 25-03-2019
Accepted: 27-04-2019

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Estimation of bioactive compound using RP-HPLC and antioxidant, antidiabetic activity of aerial parts of *Clematis heynei* and *Solanum virginianum*

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Abstract
Diabetes mellitus has become a major health concern worldwide and development and progression of diabetic related complications are accelerated by reactive oxygen species which are generated and accumulated due to hyperglycemia. Herbal remedies are convenient for the management of diabetes due to their traditional acceptability and availability, low costs and lesser side effects. The present study was carried out to determine the *in vitro* antioxidant activity, antidiabetic activity and HPLC analysis of methanol extract of *Clematis heynei* and *Solanum virginianum.* Antioxidant activities of methanolic extract of *C. heynei* and *S. virginianum* were evaluated by H_2O_2 and No scavenging assay and antidiabetic activity was determined by *in-vitro* alpha-amylase inhibition assay and quercetin was detected in methanolic extract of *C. heynei* and *S. virginianum* under study by using RP-HPLC analysis. *C. heynei* and *S. virginianum* extracts showed effective hydrogen peroxide radical scavenging and nitric oxide scavenging activity. The antioxidant activity was increased dose dependently when compared with standard drug ascorbic acid. In *in-vitro* antidiabetic test, highest α-amylase inhibition was found in methanolic extract of *C. heynei* then *S. virginianum* extract. Quercetin content was highest in *C. heynei* extract, was quantified as 0.820µg/ml and in *S. virginianum* extracts 0.798µg/ml. HPLC analysis of plant extracts indicated the presence of the most abundant dietary flavonol, Quercetin. The results of this study revealed that the bioactive compound content differences could be determinant for the medicinal properties of this plant especially for antioxidant and antidiabetic activities with potential applications in food and pharmaceutical industries.

Keywords: Diabetes mellitus, antioxidant activity, Antidiabetic activity *Clematis heynei*, *Solanum virginianum*, HPLC analysis

Introduction
Aromatic plants have been used in many domains including medicine, nutrition, flavoring, beverages, dyeing, repellents, fragrances, cosmetics, smoking and other industrial purposes. Since the prehistoric era, herbs have been the basis for nearly all medicinal therapy until synthetic drugs were developed in the nineteenth century [1, 2]. Natural products represent a rich source of biologically active compounds and are an example of molecular diversity, with recognized potential in drug discovery and development [3].Medicinal plants contain large amounts of antioxidants such as polyphenols, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. Many of these phytochemicals possess significant antioxidant capacities that are associated with lower occurrence and lower mortality rates of several human diseases [4]. Plenty of herbal extracts have been reported for their antioxidant and antidiabetic activities for the treatment of diabetes [5]. The effect of these plants may delay the development of diabetic complications and correct the metabolic abnormalities. Many new bioactive drugs isolated from plants have been shown to have hypoglycemic effect [6]. Diabetes mellitus is a complex disease characterized by gross derangement in carbohydrate, fat and protein metabolism due to deficiency in insulin secretion and/or action [7]. Mammalian α-amylase is a prominent enzyme in the pancreatic juice which breaks down large and insoluble starch molecules into absorbable molecules ultimately maltose [8]. α-glucosidase, on the other hand, anchored in the mucosal brush border of the small intestine catalyzes the end step of digestion of starch and disaccharides that are abundant in human diet [9]. Inhibitors of α-amylase and α-glucosidase delay the breakdown of carbohydrate in the small intestine and decrease the postprandial blood glucose excursion levels in diabetic patients [10]. The inhibition of these two prominent enzymes has been found as a useful and effective strategy to lower the levels of postprandial hyperglycemia [11]. *Clematis heynei* (*C. heynei*, Ranunculaceae) is commonly known as *Deccan clematis*, Murhar, Morvel, Ranjaee and it is a somewhat woody climber very sparsely

distributed in deciduous forests of Western Ghats, India. In the Indian system of medicine Ayurveda this plant is used to eliminate malarial fever and headache. Different plant parts were used for treating various diseases. Roots are given orally for secretion of bile. Leaf paste is applied externally for itches, in wounds and skin allergies [12, 13]. *Solanum virginianum* (*Solanum xanthocarpum*, Solanaceae) is an annual herb which grows as wild plant in many parts of India. In vernacular it is known as Kantakari or Bhatkatiya. Fruits are berry, yellow or with white green strips, surrounded by enlarged calyx. Fruits are edible and local people of Manipur (India) use it as folk medicine for treatment of various ailments. Irula tribes of Hasanur Hills (Tamil Nadu, India) have history of consuming the cooked unripe fruits of *S. virginianum* as vegetable [14]. In Kerala, the Kattunaikka, Paniya and Kuruma tribes of Wayanad district consume fruits and seeds as food [15]. Fruits are considered as a valuable herbal product for traditional healers in treatment of many common diseases in other parts of India. In Ayurveda, medicinal use of *S. virginianum* is well documented. Phytoconstituents present in *S. virginianum* are used as anti-fertility, anti-inflammatory, anti-allergic agents and as potential fungicide [16, 17]. In the present study, quantification of quercetin by HPLC technique has been made in relation to its antioxidant and antidiabetic activity which was not investigated previously.

Material and method

Plant material

Whole plant material of *C. heynei and S. virginianum* were collected from local area of Bhopal (M.P.) in the month of August, 2017.

Chemical reagents

All the chemicals used in this study were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), SigmaAldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India). All other chemicals and solvents used were of HPLC and analytical grade.

Extraction Procedure

Defatting of plant material

C. heynei and *S. virginianum* were shade dried at room temperature. The shade dried plant material was coarsely powdered and subjected to extraction with petroleum ether by maceration. The extraction was continued till the defatting of the material had taken place.

Extraction

100gm of dried plant material (both plant each separately) were exhaustively extracted with different solvent (chloroform, ethyl acetate, methanol, aqueous) using maceration method for 48 hrs. Filtered and dried using vacuum evaporator at 40 ^{0}C. Finally the percentage yields were calculated of the dried extracts [18].

Qualitative and quantitative phytochemical analysis of plant extract

In vitro antioxidant activity

The methanol extract of *C. heynei and S. virginianum* was tested for its in vitro antioxidant activity using the standard methods. In all these methods, a particular concentration of the extract or standard solution was used which gave a final concentration of 10-500 µg/ml after all the reagents were added. Absorbance was measured against a blank solution containing the extract or standards, but without the reagents. A control test was performed without the extract or standards. Percentage scavenging and IC50 values ± SEM were calculated.

Hydrogen peroxide radical scavenging method

In this method, when a scavenger is incubated with hydrogen peroxide, the decay or loss of hydrogen peroxide can be measured spectrophotometrically at 230 nm. A volume of 2 ml of hydrogen peroxide (20 mM) in phosphate buffer saline (PBS, pH 7.4) was added to 1 m of various concentrations of extract or standard (20to100µg/ml) in ethanol. After 10 min the absorbance was measured at 230 nm against a blank solution that contained hydrogen peroxide solution without the extract [19].

The percentage of H_2O_2 scavenging of the plant extract was calculated as follows:

% scavenged [H_2O_2] = [(Abs control–Abs sample)/Abs control] ×100

Nitric oxide radical inhibition assay

Nitric oxide was generated from sodium nitroprusside and measured by the Griess reaction. Sodium nitroprosside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitric ions that can be estimated by using Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide. The reaction mixture (6 m) contained sodium nitroprusside (10 mM, 4 ml), phosphate buffer saline (PBS,pH7.4,1ml) and extract or standard (1 ml, 10-200 µg/ml) in DMSO at various concentrations and it was incubated at 25±2 ºC for 150min. After incubation, 0.5ml of the reaction mixture containing nitrite ion was removed, sulphanilic acid reagent was added (0.33% w/v, 1ml), mixed well and allowed to stand for 5 min for completion of diazotisation. Then, 1 ml of NEDD was added, mixed and allowed to stand for 30 min in diffused light. A purple colored chromophore was formed. The absorbance was measured at 546 nm [19]. The Nitric oxide radical scavenging ability was calculated using the following equation:

$$\text{Scavenging activity (\%)} = \frac{A_0 - A_t}{A_0} \text{X } 100$$

Where A_0 is the absorbance of the control and A_t is the absorbance of the sample.

In vitro anti diabetic activity

α-amylase Inhibitory (AAI) Assay

A total of 500 µl of test samples and standard drug (10-50µg/ml) were added to 500 µl of 0.20 mM phosphate buffer (pH 6.9) containing α-amylase (0.5mg/ml) solution and were incubated at 25 °C for 10 min. After these, 500 µl of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added to each tube. The reaction mixtures were then incubated at 25 °C for 10 min. The reaction was stopped with 1.0 ml of 3, 5 dinitrosalicylic acid (DNS) solution. The test tubes were then incubated in a boiling water bath for 5 min, cooled to room temperature. The reaction mixture was then diluted after adding 10 ml distilled water and absorbance was measured at 540 nm. Control represent 100% enzyme activity and were conducted in similar way by replacing extract with vehicle. The experiments were repeated thrice using the same protocol [20].

Quantification of flavonoid compounds by HPLC technique

For HPLC investigation of flavonoid compounds the methanolic extracts of *C. heynei and S. virginianum* under study were used as a preliminary assessment of various compounds. The HPLC apparatus used for analysis was composed of a waters equipped with a UV dual detector and generated data were analyzed using Waters Ace software. For chromatographic separation Thermo C18 column (250X4.6mm, 5µm) was applied. The chromatographic analysis was performed at ambient temperature on a RP-C18 analytical column with a mobile phase composed of Acetonitrile: Methanol (50:50 v/v) and was isocratically eluted at a flow rate of 1 mL/ min. A small sample volume of 20 µL was used for each sample run, being injected into the HPLC system. The chromatogram was monitored with UV detection at a wavelength of 256 nm. Analysis time was 15min for both, standards and samples used for analysis. A quercetin was used as standards. A thermospectronic model of Labindia 3000 + UV/VIS Spectrophotometer with 1cm. matched quartz cells was used for determination of λmax.

Results and discussion

The crude extract so obtained after the maceration extraction process, extract was further concentrated on water bath evaporation the solvent completely to obtain the actual yield of extraction. To obtain the percentage yield of extraction is very important phenomenon in phytochemical extraction to evaluate the standard extraction efficiency for a particular plant, different parts of same plant or different solvents used. The yield of extracts obtained from different samples using Pet. ether, chloroform, ethyl acetate, methanol, aqueous as solvents are depicted in the table 1. The results showed that maximum yield was found in alcoholic extract. It may due to the solubility of principle contents presence be higher in case of alcoholic solvent, thus it has been accepted that it is a universal solvent for the extraction of plant constituents.

Table 1: Results of percentage yield of plant material

Solvents	*C. heynei*	*S. virginianum*
Pet ether	1.8%	3.1%
Chloroform	2.4%	2.1%
Ethyl acetate	4.1%	3.6%
Methanol	4.3%	4.6%
Aqueous	3.6%	3.4%

Free radicals are chemical species which contains one or more unpaired electrons. They are highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability. They are formed inside the system, and are highly reactive and potentially damaging transient chemical species. These radicals are continuously produced in the human body because they are essential for detoxification, chemical signaling, energy supply and immune function. Free radicals are regulated by endogenous antioxidant enzyme system, but due to over production of free radicals by exposure to environmental oxidant substances such as cigarette smoking, UV radiation, etc or a failure in antioxidant defense mechanism or damage to cell structures, the risk increases for many diseases such as Alzheimer's disease, mild congestive impairment, Parkinson's disease, cardiovascular disorders, liver diseases, ulcerative colitis, inflammation and cancer [21]. Interestingly, the body possesses defense mechanisms against free radical induced oxidative stress, which involve preventive and repair mechanisms, i.e. physical defense and antioxidant defense. Hydrogen peroxide itself is not very reactive, but sometimes it is toxic to cell because it may give rise to hydroxyl radical in the cells. Therefore, removing of hydrogen peroxide is very important for antioxidant defense in cell system. Polyphenols have also been shown to protect mammalian cells from damage induced by hydrogen peroxide, especially compounds with the orthohydroxy phenolic compounds like quercetin, gallic acid, caffeic acid and catechins [22]. Therefore, the phenolic compounds of the *C. heynei* and *S. virginianum* extract may probably be involved in scavenging hydrogen peroxide. Nitric oxide formed during their reduction with oxygen or with superoxide, such as NO_2, N_2O_4, N_3O_4 is very reactive. These radicals are responsible for altering the structure and functional behavior of many cellular components. The *C. heynei* extract showed better activity in competing with oxygen to react with nitric oxide and thus inhibited the generation of anions and the activity is comparable to the standards used. The plant secondary metabolites may have the property to counteract the effect of nitric oxide formation and in turn may be considerably interested in preventing the ill effects of excessive nitric oxide generation in the human body. Further the scavenging activity may also help to arrest the chains or reactions initiated by excess generation of nitric oxide that are dangerous to the human health. Nitric oxide is also implicated for inflammation, cancer and other pathological conditions [23]. Nitric oxide radical scavenging activity of *C. heynei* and *S. virginianum* extract was shown in Table 2. The extract exhibited potent radical scavenging activity in concentration dependent manner. The IC50 value of the extracts was comparable to the standards used. In hydrogen peroxide radical scavenging assay, the extract was found to be less potent when compared to ascorbic acid. The values were tabulated in Table 3. In both assay method methanolic extract of *C. heynei* was more potent than *S. virginianum* extract but less potent than standard ascorbic acid.

Table 2: Results of nitric oxide radical scavenging assay

Conc.	Ascorbic acid	Methanolic Extract	
	% Inhibition	*C. heynei*	*S. virginianum*
20	48.59438	34.73896	17.26908
40	56.82731	37.7510	29.31727
60	60.24096	49.39759	37.3494
80	76.90763	76.90763	57.02811
100	80.32129	80.32129	76.90763
IC 50	25.15588	51.12135	68.83106

Absorbance of control (Ao) = 0.498

Table 3: Results of hydrogen peroxide scavenging assay

Conc.	Ascorbic acid	Methanolic extract	
	% Inhibition	*C. heynei*	*S. virginianum*
20	45.06699	32.52132	20.34105
40	60.41413	44.70158	32.52132
60	61.99756	61.99756	44.21437
80	82.33861	79.90256	60.90134
100	88.06334	85.38368	73.81242
IC 50	27.45	44.573	65.442

Absorbance of control (Ao) = 0.821

Pancreatic α-amylase, as a key enzyme in the digestive system, is involved in the breakdown of starch into disaccharides and oligosaccharides and finally liberating glucose which is later absorbed into the blood circulation. Inhibition of α-amylase would diminish the breakdown of starch in the gastro-intestinal tract. Therefore, the postprandial

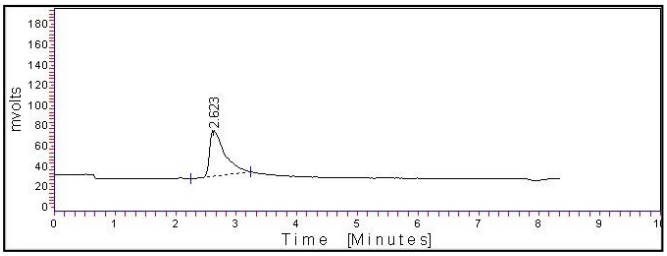
hyperglycemia level may also be reduced [24]. The inhibition of α-amylase enzyme activity of *C. heynei* and *S. virginianum* extract and standard drug acarbose is presented in table 4. Obtained results revealed that the methanolic extracts inhibit α-amylase enzyme in a dose-dependent manner (100-500µg/ml). Compared with the standard acarbose, the methanolic extract of *C. heynei* plant sample showed significant inhibition activity (161.78%). Shobana *et al*. [25] reported that the phenolic-rich plant extracts have higher ability to inhibit α-amylase enzyme. Plant-derived phenolics and natural antioxidants are recently warranted because of their less severe side effects [26].

Table 4: Results of *In vitro* antidiabetic studies

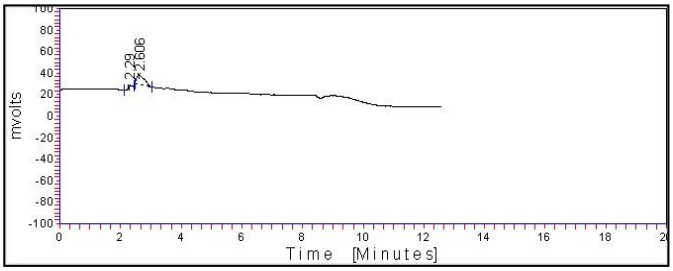
	Acarbose	*C. heynei*	*S. virginianum*
Conc.	**% Inhibition**	**% Inhibition**	**% Inhibition**
100	47.13467	43.40974	34.38395
200	67.62178	54.01146	54.01146
300	79.22636	62.03438	67.76504
400	85.95989	76.36103	82.09169
500	90.68768	77.65043	85.95989
IC_{50} (µg/ml)	71.53	161.78	187.26

Control Absorbance = 0.698

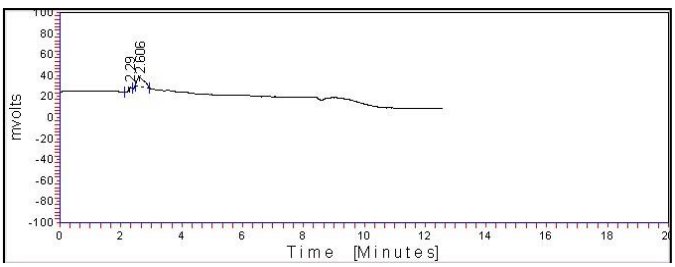
The HPLC chromatogram of standard quercetin and hydroalcoholic extract are shown in Figure 1 and the values are expressed in ppm. The retention time for standard and extracts was found to be 2.623 min and 2.606 min respectively. Characteristics parameters for standard quercetin was given in table 5 and results of quantitative estimation of quercetin in methanolic extracts were given in table 6.



(A)



(B)



(C)

Fig 1: Chromatogram of (A) Standard quercetin (B) Methanolic extract of *C. heynei* (C) Methanolic extract of S. *virginianum*

Table 5: Characteristics of the analytical method derived from the std. calibration curve

Compound	Linearity range µg/ml	Correlation co-efficient	Slope	Intercept
Quercetin	5-25	0.999	25.25	-1.451

Table 6: Quantitative estimation of quercetin in extract

Extract	RT	Area	% Assay
C. heynei	2.606	177.165	0.820
S. virginianum	2.606	157.145	0.798

Conclusion

Presence of bioactive polyphenolic compounds is responsible for the medicinal properties of plants. Both plants have been used as a source of phytomedicine due to the presence of considerable amount of phenols and flavonoids. Due to presence of phenols and flavonoids, the extract showed significant amount of antioxidant activity. This plant also reveals better in vitro enzyme inhibitory activity (alpha amylase) which is involved in regulation and absorption of carbohydrate. The study suggests that the aerial parts of the plant might be a potential source of natural antioxidants. Thus *C. heynei* and *S. virginianum* can be a good candidate for novel phytomedicine that can be used to treat several diseases. The future study shall be directed towards the identification of bioactive compounds and quality standards for developing a potential drug.

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