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Extraction, Qualitative and Quantitative Determination of Secondary Metabolites of Aerial Parts of *Clematis heynei* and *Solanum virginianum*

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ABSTRACT

The increasing interest in powerful biological activity of secondary metabolites outlined the necessity of determining their contents in medicinal plants. In the last few years, there has been an exponential growth in the field of herbal medicine and gaining popularity both in developing and developed countries because of their natural origin and less side effects. *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. *Solanum virginianum* L. (*S. virginianum,* Solanaceae, Solanum xanthocarpum Schrad. & H. Wendl.) is a diffuse and very prickly under shrub. It is found growing commonly in various regions of the world on sandy soils and is distributed throughout India. The plant is used traditionally to treat asthma, chest pain, leucoderma, scorpion bite, and sterility in women. The aim of the present study is to examine *C. heynei* and *S. virginianum* aerial parts of plant for phytochemical profile. Qualitative analysis of various phytochemical constituents and quantitative analysis of total phenolics and flavonoids were determined by the well-known test protocol available in the literature. Quantitative analysis of phenolic and flavonoids was carried out by Folins Ciocalteau reagent method and aluminium chloride method respectively. Phytochemical analysis revealed the presence of phenols, flavonoids, tannins, saponins, alkaloids, fixed oil and fats. The total phenolics content of methanolic and aqueous extract *of C. heynei* was (0.592, 0.292 mg/100mg), followed by flavonoids (1.371, 0.723mg/100mg) respectively. The total phenolics content of methanolic extract of *S. virginianum* was (0.345mg/100mg), followed by flavonoids (0.978mg/100mg). The present study concluded that the crude extract of *C. heynei* and *S. virginianum* is a rich source of secondary phytoconstituents which impart significant antioxidant potential. The findings of the present study will be helpful to phytochemists, pharmacologists and pharmaceutical industries.

Keywords: *Clematis heynei, Solanum virginianum,* Phytochemical, Folins ciocalteau reagent

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INTRODUCTION

Herbal medicines have become more popular in the treatment of many diseases due to the popular belief that green medicine is safe, easily available and has fewer side effects. Secondary metabolites of plants serve as defense mechanisms against predation by many microorganisms, insects and herbivores [1]. Phytochemical composition and respective biological activities are important to understand the therapeutic potential of medicinal herbs. Among other, phenolic compounds are the most widely explored phytochemicals for therapeutic potential in different medicinal plants. Most of these studies conclude that pharmacological activities of any medicinal plant are due to the presence of secondary metabolites. Secondary metabolites usually consist of the phenolic compounds, alkaloids, tannins, saponins, carbohydrates, glycosides, flavonoids, steroids, etc. Most phenolic compounds such as flavonoids, glycosides, triterpenoids, flavonons, carbohydrates and anthraquinones are commonly present in most of the medicinal plants. All of these secondary metabolites and particularly phenolic compounds have been reported as scavengers of free radicals and also have been considered as good therapeutic candidates for free radical related pathologies [2]. Several plants have been studied for quantification of secondary metabolites, such as *Jatropha* [3], *Clerodendron colebrookianum* and *Zingiber cassumunar* [4], *Spondias mombin*[5] and leaves of *S. hyderobadensis* [6]. *S. virginianum L.* is a diffuse and very prickly under shrub belonging to the family Solanaceae It is found growing commonly in various regions of the world on sandy soils and is distributed throughout India. It is commonly called as yellow-berried nightshade in English, kantakari in Sanskrit and nelagulla in Kannada. It is one of the members of

dashamula of Ayurveda [7]. A wide range of phytochemicals such as alkaloids, phenolics, flavonoids, sterols, saponins, glycosides, fatty acids, tannins, and amino acids have been identified from different parts of the plant. The plant is extensively used in various systems of medicine including Ayurveda. The plant is used traditionally to treat asthma, chest pain, leucoderma, scorpion bite, and sterility in women. Roots are much used in medicine. The oil from seeds is used to treat arthritis. The ash from dried fruits is used to relieve toothache [8-11]. The plant is shown to exhibit various bioactivities such as antimicrobial, anthelmintic, antioxidant, hemolytic, anti-inflammatory, antidiabetic, cytotoxic, phytotoxic, hepatoprotective, and immunostimulatory activities [12]. The medicinal plant *C. heynei* is a member of the Ranunculaceae (buttercup) family. 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. Roots are given orally for secretion of bile. Leaf paste is applied externally for itches, in wounds and skin allergies. The traditional medicine practitioners give the root decoction orally or small pieces placed in mouth in bilious vomiting. Leaf juice for treating boils leprosy, blood diseases and cardiac disorders. Decoction of root is given every night with boiled rice water to the children as anthelmintic [13]. Flower juice applied externally for acne, black heads and black spots on face [14]. Clematis species has many different pharmacological effects such as antibacterial, anti-inflammatory, antitumor, analgesic and diuretic functions [15]. The juice of the leaves, combined with that of the leaves of *Holarrhena antidysenterica*, is dropped into the eye for the relief of pain in staphyloma; about 2 drops being used. Reports on the chemical components of genus clematis have been scarce up to now and mainly refer to triterpenoid saponins [15-18]. Many investigators successfully isolated some of the secondary metabolites from the species of clematis. Clemontanoside-C, a new hedragenin based saponin was isolated from the stem of *C. montana* [19]. From the aerial part of *C. tibetana*, two new hedragenin 3, 28-O-bisdesmosides called clematibetosides A and C, and a new gypsogenin 3, 28-O-bisdesmoside called clematibetoside B have been isolated [20]. Protoanemonin has been isolated from the Australian 'Headache Vine' *C. glycinoides* [21]. Though the plant has been reported for many biological activities, no scientific data available to identify the genuine sample. The aim of this work was to determine the quality (types), quantity (amount) of bioactive compounds of aerial parts of *C. heynei* and *S. virginianum*.

MATERIAL AND METHOD

Plant material

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

Chemical reagents

All the chemicals used in this study were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), Sigma Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals used in this study were of 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 vaccum evaporator at 40°C. Finally the percentage yields were calculated of the dried extracts [22].

Qualitative phytochemical analysis of plant extract

The *C. heynei* and *S. virginianum* extracts obtained was subjected to the preliminary phytochemical analysis following standard methods by Khandelwal and Kokate [23, 24]. The extract was screened to identify the presence or absence of various active principles like phenolic compounds, carbohydrates, flavonoids, glycosides, saponins, alkaloids, fats or fixed oils, protein and amino acid and tannins.

Test for carbohydrates

Molisch's test: In a test tube containing extract of drug, added two drop of freshly prepared 20% alcoholic solution of α- napthol and mixed concentrated sulphuric acid along the sides of the test tube. If carbohydrate present purple color or reddish violet color produce at the junction between two liquids.

Benedict's test: In a test tube containing extract of drug add benedict's solution, mix well, boiled the mixture vigorously for two minutes and then cooled. Formation of red precipitate due to presence of carbohydrates.

Barfoed's test: The barfoed's solution added to 0.5 ml of solution under examination, heated to boil. Formation of red precipitate of copper oxide was indicated the presence of carbohydrates.

Anthrone test: To the two ml of anthrone test solution, add the extract of drug. A green or blue colour indicated the presence of carbohydrate.

Test for alkaloids

Dragendorff's Test: Few mg of extract of the drug dissolved in 5 ml of water added 2 M hydrochloric acid until an acid reaction occurred; 1 ml of dragendorff's reagent (potassium bismuth iodide solution) was added an orange red precipitate indicated the presence of alkaloids.

Wagner's test: Acidify the extract of drug with 1.5 % v/v of hydrochloric acid and added a few drop of Wagner's reagent (iodine potassium iodide solution). Formations of reddish brown precipitate indicated the presence of alkaloids.

Mayer's Test: Two ml of extract solution was treated with 2 - 3 drops of Mayer's reagent was added (potassium mercuric iodide solution) formation of dull white precipitate indicated the presence of alkaloid.

Hager's Test: Extract of the drug solution was treated with 3 ml of Hager's reagent (saturated solution of picric acid) formation of yellow precipitate confirmed the presence of alkaloids.

Test for glycosides

Legal's test: Extract solution dissolved in pyridine then sodium nitroprusside solution was added to it and made alkaline. Pink red colour indicated the presence of glycosides.

Baljet's test: To the drug extract, sodium picrate solution was added, yellow to orange colour was indicated the presence of glycosides.

Borntrager's test: Few ml of dilute sulphuric acid solution, the test solution of extract was added. It was filtered and the filtrate was boiled with ether or chloroform. Then organic layer was separated to which ammonia was added, pink, red or violet colour was produced in orange layer confirmed the presence of glycosides.

Keller Kiliani test: Methanolic extract was dissolved in glacial acetic acid containing trace of ferric chloride one ml concentrated sulphuric acid was added carefully by the side of the test tube. A blue colour in the acetic acid layer and red colour at the junction of the two liquid indicated the presence of glycosides.

Test of saponins

1 ml of alcoholic extract was diluted with 20 ml distilled water and shaken in graduated cylinder for 15 minutes. One cm layer of foam indicated the presence of saponins.

Test for flavonoids

Shinoda test: In the test tube containing alcoholic extract of the drug added 5 - 10 drops of dil. hydrochloric acid followed by the small piece of magnesium. In presence of flavonoids a pink, reddish pink or brown color was produced.

Test for tannins

To the sample of the extract, ferric chloride solution was added appearance of dark blue or greenish black colour indicated the presence of tannins.

To the sample of extract, potassium cyanide was added, deep red colour was confirmed the presence of tannins.

To the sample of extract, potassium dichromate solution was added, yellow precipitate was produced.

Test for protein and amino acid

Biuret's test: To 2 - 3 ml of the extract of drug added in 1 ml of 40 % sodium hydroxide solutions and 2 drops of 1 % copper sulphate solution mix thoroughly, a purplish - violet or pinkish - violet colour produced that indicates the presence of proteins.

Ninhydrin's test: Two drops of freshly prepared 0.2 % ninhydrin reagent was added to the extract and heated to boiling for 1 - 2 min. and allow cooling. A blue colour developed that indicating the presence of proteins, peptides or amino acids.

Xanthoprotein test: To the extract in a test tube, add conc. nitric acid. A white precipitate was obtained and upon heating turns to yellow and cool the solution carefully. Added 20 % of sodium hydroxide solution in excess orange colour indicated presence of aromatic amino acid.

Millon's test: The small quantity of extract of the drug dissolved in distilled water added 5 - 6 drop of millon's reagent. A white precipitate was formed which turned red on heating, indicated the presence of proteins.

Lead acetate test: The extract was taken and two ml of 40 % sodium hydroxide solution was added and boiled, glacial acetic acid was added and cooled than added 1 ml of lead acetate solution, gray black precipitate was formed which indicated presence of sulphur containing amino acid.

Test of fats or fixed oils

Using sodium hydroxide: The extract was mixed in one ml 1 % of copper sulphate solution then added 10 % sodium hydroxide solution a clear blue solution was obtain which showed glycerin present in sample.

Using sodium hydrogen sulphate: The extract was taken in test tube added a pinch of sodium hydrogen sulphate pungent odour was formed which showed glycerin present in sample.

Saponification: Four ml of 2 % sodium carbonate solution was taken and the extract was added. Shaked vigorously and boiled it. A clean soapy solution was formed cooled and added few drops of conc. HCl and observed that fatty separate out and float up.

Quantification of secondary metabolites

Quantitative analysis is an important tool for the determination of quantity of phytoconstituents present in plant extracts. For this TPC and TFC are determined. Extracts obtained from aerial parts of *C. heynei* and *S. virginianum* plant material of subjected to estimate the presence of TPC and TFC by standard procedure.

Total phenol determination

The total phenolic content was determined using the method of Olufunmiso *et al* [25]. A volume of 2ml of each extracts or standard was mixed with 1 ml of Folin Ciocalteau reagent (previously diluted with distilled water 1:10 v/v) and 1 ml (7.5g/l) of sodium carbonate. The mixture was allowed to stand for 15 min under room temperature. The colour developed was read at 765 nm using UV/visible spectrophotometer. The total phenolic content was calculated from the standard graph of gallic acid and the results were expressed as gallic acid equivalent (mg/100mg).

Total flavonoids determination

The total flavonoid content was determined using the method of Olufunmiso *et al* [25]. 1ml of 2% $AlCl_3$ methanolic solution was added to 3 ml of extract or standard and allowed to stand for 15 min at room temperature; the absorbance of the reaction mixture was measured at 420 nm using UV/visible spectrophotometer The content of flavonoids was calculated using standard graph of quercetin and the results were expressed as quercetin equivalent (mg/100mg).

RESULTS AND DISCUSSIONS

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%

The results of qualitative phytochemical analysis of the crude powder of aerial parts of *C. heynei* and *S. virginianum* are shown in Table 2 &3. Methanolic and aqueous extracts of *C. heynei* and *S. virginianum* showed the presence of flavonoids, phenols, saponins and diterpins. But chloroform and ethyl acetate extracts show the presence of saponins and diterpins.

Table 2: Phytochemical screening of *C. heynei* extracts

Constituents	Chloroform	Ethyl acetate	Methanol	aqueous
Alkaloids				
Dragendroff's test	-ve	-ve	-ve	-ve
Hager's test	-ve	-ve	-ve	-ve
Glycosides				
Legal's test	-ve	-ve	-ve	-ve
Flavonoids				
Lead acetate	-ve	-ve	+ve	+ve
Alkaline test	-ve	-ve	+ve	+ve
Phenolics				
$Fecl_3$	-ve	-ve	+ve	+ve
Proteins And Amino acids				
Xanthoproteic test	-ve	+ve	-ve	-ve
Carbohydrates				
Fehling's test	-ve	-ve	-ve	-ve
Saponins				
Foam test	-ve	+ve	+ve	+ve
Diterpins				
Copper acetate test	+ve	+ve	+ve	+ve

Table 3: Phytochemical screening of *S. virginianum* extracts

Constituents	Chloroform	Ethyl acetate	Methanol	aqueous
Alkaloids				
Dragendroff's test	-ve	-ve	-ve	-ve
Hager's test	-ve	-ve	-ve	-ve
Glycosides				
Legal's test	-ve	-ve	-ve	-ve
Flavonoids				
Lead acetate	-ve	+ve	+ve	-ve
Alkaline test	-ve	+ve	+ve	-ve
Phenolics				
$Fecl_3$	-ve	-ve	+ve	-ve
Proteins And Amino acids				
Xanthoproteic test	-ve	+ve	+ve	+ve
Carbohydrates				
Fehling's test	-ve	+ve	+ve	-ve
Saponins				
Foam test	-ve	+ve	+ve	+ve
Diterpins				
Copper acetate test	+ve	+ve	+ve	+ve

The determination of the total phenolic content, expressed as mg gallic acid equivalents and per 100 mg dry weight of sample. The total flavonoids content of the extracts was expressed as percentage of quercetin equivalent per 100 mg dry weight of sample. TPC and TFC of methanolic and aqueous extract of *C. heynei* showed the content values of 0.592, 0.292 and 1.371, 0.723 respectively. But chloroform and ethyl acetate extracts of *C. heynei* have no TPC and TFC. The total phenolic and flavonoid content of *S. virginianum* methanolic extracts showed the content values of 0.345and 0.978 respectively. Results are provided in table 4&5.

Table 4: Total phenolic and flavonoid content *C. heynei* extracts

Extracts	Total Phenol (mg/100mg)	Total flavonoid (mg/100mg)
Methanolic	0.592	1.371
Aqueous	0.292	0.723

Table 5: Total phenolic and flavonoid content *S. virginianum* extracts

Extracts	Total Phenol (mg/100mg)	Total flavonoid (mg/100mg)
Methanolic	0.345	0.978

CONCLUSION

Qualitative and quantitative analysis of phenolics and flavonoids from *C. heynei and S. virginianum* was achieved first time in this work. The observed level of phytoconstituents revealed that *C. heynei and S. virginianum* is a rich source of antioxidant compounds. Currently available synthetic antioxidants are suspected to cause or prompt negative health effects, hence strong restrictions have been placed on their application and there is a trend to substitute them with naturally occurring antioxidants. Moreover, the plant parts may be used as an alternative source for flavonoids and phenols for traditional remedies. Further phytochemical studies are also required to isolate and characterize active ingredients that are responsible for its antioxidant activity and to explore the existence of synergism if any, among the compounds.

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