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Original Article

PRELIMINARY PHYTOCHEMICAL, PHYSICOCHEMICAL, ANTIOXIDANT AND ANTIMICROBIAL STUDIES OF *CLEMATIS MONTANA* LEAVES

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ABSTRACT

Objective: To evaluate the phytochemical, physicochemical, antimicrobial and *in-vitro* antioxidant activity of *Clamentis montana* (Family: Rananculaceae).

Methods: The extracts were evaluated for antibacterial activity against *S. aureus, B. subtilis, E. coli, P. aeruginosa* by cup plate method. In-vitro antioxidant activity was done by DPPH, ferrous chelating and reducing power assay method. The physicochemical parameter like a loss on drying, total ash value, foreign matter, etc. was evaluated by standard protocol.

Results: The extract showed significant antibacterial activity against all test strains when compared with standard drugs amoxicillin. The extract showed significant antioxidant activity by DPPH method, reducing power assay and ferrous chelating method.

Conclusion: The extract showed a dose-dependent significant antibacterial and antioxidant activity.

Keywords: *Clematis montana*, Antibacterial, Antioxidant, Ethanolic extract

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INTRODUCTION

Clematis is a genus of climbing vines with showy flowers, and over 200 species are known. They are famous as flowering plants. *Clematis montana* belongs to the family Ranunculacece. *Clematis montana* found in Gharwal Himalaya region of Uttarakhand. *Clematis montana* had many medicinal properties and used for the treatment of a migraine, nervous disorders, skin infections, liver complications, hypertension and diabetes [1, 2]. Clemontanoside F (triterpene bis glycoside) and three saponin {Clemontanoside-C, [(3-0-β-ribopyranosyl) (l-3)-α-rhamnopyranosyl (l-2)-α-arabino-pyranosido-28-0-α-L-rhamnopyranosyl (l-4)-β-D-gluco-pyranosyl (l-6)-β-D-glucopyranoside of hederagenin)] [(3-0-β-ribopyranosyl (l-3)-α-rhamnopyranosyl-(l-2)-a-arabinopyranoside of hedera-genin)]} had been isolated from *Clematis Montana* [3-5]. As not much work has been done on antibacterial activity, it was considered worthwhile to carry out this activity together with antifungal activity.

Free radicals are types of reactive Oxygen Species, which include all highly reactive, oxygen-containing molecules. Types of reactive Oxygen Species include the hydroxyl radical, the superoxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical, and various lipid peroxides. These free radicals may either be produced by physiological or biochemical processes or by pollution and other endogenous sources. All these free radicals react with basic cell structure like membrane lipid, DNA, RNA, enzymes, etc. and damage the cell [6]. These free radicals are responsible for the development of chronic diseases such as diabetes, cancer, hypertension, cardiac infarction, arteriosclerosis, rheumatism, cataracts and others [7]. Natural antioxidants play an important role to neutralize the free radicals. Several epidemiological, clinical and experimental data suggest that plant-based antioxidants have beneficial effects on prevention on chronic diseases [8, 9]. Plants are a rich source of free radical scavenging molecules such as vitamins, terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains and other metabolites which are rich in antioxidant activity [10].

MATERIALS AND METHODS

Collection of plant material

The whole plant of *Clemantis montana* was collected from Dhanaulti, Mussorie Uttarakhand, India during Oct-Nov and was authenticated at Department of Botany H. N. B Garhwal University, Srinagar Garhwal, Uttarakhand by taxonomist Dr. J. K. Tewari.

Preparation of extract

The fresh leaves were dried at room temperature (25-30°C) for 20 d and powdered. Six hundred gram of coarse powder was extracted with methanol in a soxhlet extractor for 72 h. The solvent from total extract was distilled off and concentrated on a water bath to syrupy consistency and then evaporated to dryness. The yield was found to be 8.98%.

Preliminary phytochemical screening

The qualitative chemical tests of methanolic extract of *C. montana* were carried out by using standard procedure to determine the presence of various phytochemicals [11].

Physicochemical constants

Total ash value was determined by taken accurately weighed 2 g of sample extract into ignited tared silica crucible where it was spread like a fine layer on the bottom. At the increasing temperature, the sample was burnt up to red hot not exceeding 450 °C until free from carbon. Then the crucible was cooled, and resultant ash was weighed, and the thereupon percent total ash value was determined with reference to air dried extract drug. The obtained ash during the above procedure was taken and boiled with 2N HCl (25 ml) for 5 min respectively for quantitative estimation of acid insoluble ash. Thereafter the insoluble ash was recovered on an ash less filter paper and washed using hot water. The insoluble sample was transferred into a crucible which was again burnt for 20 min and weighed properly. In order to omit errors the whole step was repeated thrice and the percent acid insoluble ashes were determined with reference to air-dried drug. Water soluble ash was determined by using the recovered ash during the estimation for

total ash was taken and boiled with water (25 ml) for 5 min interval. Thereafter the insoluble ash was recovered on an ash less filter paper and washed using hot water. The insoluble sample was transferred into a crucible which was again burnt for 20 min and weighed properly. The whole step was repeated thrice in order to omit errors and the percent water soluble ashes were determined with reference to air-dried drug respectively [12-14].

Loss on drying

This parameter determines the amount of moisture as well as volatile components present in a particular sample (i.e. water drying off from the drug). The powdered drug sample (10 gm) without preliminary drying was placed on a tarred evaporating dish and dried at 105 °C for 6 h and weighed. The drying was continued until two successive reading matches each other or the difference between two successive weighing was not more than 0.25%. Constant weight was reached when two consecutive weighing after drying for 30 min in a desiccator, showed not more than 0.01 gm difference [15].

Foreign matter

It is the matter present in the drug. Its presence may be due to a faulty collection of crude drug or due to deliberate mixing. It was separated from the drug so that results obtained from analysis of the drug gives accuracy. Its percentage in the crude drug was calculated.

***In vitro* antioxidant studies**

(a) DPPH method

Preparation of extract standard dilution

25 mg of ethanolic extract of leaves was weighed and dissolved in 25 ml of methanol to get 1000µg/ml stock solution. Lower concentrations (100, 200, 300, 400 µg/ml) was prepared by diluting serially with methanol. Ascorbic acid was weighed (10 mg) and dissolved in 100 ml methanol to get 100 µg/ml stock solutions. Lower concentrations (2, 4, 6, 8, 10 µg/ml) was prepared by diluting serially with methanol.

Method

The stable 1, 1-diphenyl-2-picryl hydroxyl radical (DPPH) was used for determination of free radical-scavenging activity of the extract. 50 mmol (0.00147 gm in 50 ml) solution of DPPH in methanol was freshly prepared. Different concentrations of extract and standard were added to the equal volume of a methanolic solution of DPPH. After 30 min at room temperature, the absorbance was recorded at 517 nm. A control reaction was carried out without the test sample. IC_{50} values denote the concentration of the sample, which is required to scavenge 50% of DPPH free radicals. Radical scavenging activity was calculated by the following formula

$$\% \text{ Inhibition} = (A_0 - A_1)/A_0 \times 100$$

where A_0 is the absorbance of control and A_1 is the absorbance of extract or standard.

IC_{50}value was determined from the plotted graph of scavenging activity against the different concentrations of C. montana extracts, which is defined as the concentration of antioxidant necessary to decrease the DPPH radical concentration by 50 %. The measurements were carried out three times, and their scavenging effect was calculated based on the percentage of DPPH scavenged [16].

(b) Ferrous chelating activity

1 ml of each dilution of ethanolic extract (200-1000 µg/ml) and standard BHT (100-400 µg/ml) was added to a solution of 2 mmol ferrous chlorides (0.2 ml). The reaction was initiated by the addition of 5 mmol ferrozine (0.4 ml). The total volume was adjusted to 4 ml with ethanol. Then, the mixture was shaken vigorously and kept at room temperature for 10 min. The absorbance of the solution was measured spectrophotometrically at 562 nm [17].

(c) Reducing power

1 ml of ethanolic extract (100-400 µg/ml), standard ascorbic acid dilutions (20-100 µg/ml) and control sample (1 ml distilled water instead of sample solution) was mixed with 2.5 ml phosphate buffer solution (pH 6.6) and 2.5 ml potassium ferricyanide (1%). The final mixture was properly mixed and incubated at 50 °C for 20 min. After incubation, the reaction mixture was rapidly cooled and mixed with 2.5 ml of 10% trichloroacetic acid. It was centrifuged at 3000 rpm for 10 min. About 2.5 ml of the supernatant was taken, and 2.5 ml distilled water and 0.5 ml of ferric chloride (0.1%) were added, it was mixed well and allowed to stand for 10 min. The absorbance was measured at 700 nm [18].

Antimicrobial activity

The ethanolic extract of leaves was examined for antibacterial activity against Gram-positive bacteria *S. aureus, B. subtilis*, Gram-negative bacteria *E. coli, P. aeruginosa* and antifungal activity against *C. albicans*. The antimicrobial screening was performed by agar well diffusion method. Muller Hinton Agar medium (Hi-media) and Sabouraud agar medium were used for bacterial and fungal strains respectively. The mediums were sterilized by autoclaving at 120 °C for 25 min and poured into Petri-plates and allowed to solidify. The plates were swabbed with the bacterial strains of *S. aureus, B. subtilis, E. coli, P. aeruginosa* and fungal strain of *C. albicans.* In each plate wells of 6 mm diameter were made at equal distances using sterile cork borer. Different dilutions of the extract were made having concentration of 100µg/ml, 250µg/ml, 500µg/ml, and 1000µg/ml in DMSO (dimethyl sulphoxide). 0.1 ml of each test solution and control were placed in 6 mm diameter wells. One well was filled with 0.1 ml of standard drug Amoxycillin (10 µg/ml) in the case of antibacterial activity whereas standard drug Fluconazole (10 µg/ml) in antifungal activity. The Petri plates were incubated at 37 °C for 24 h for antibacterial and at about 28 °C for 48 h for antifungal activity respectively. The diameter of the zone of inhibition was measured [19]. The diameter obtained for the test samples were compared with diameter produced by the standard Amoxycillin and fluconazole in antibacterial and antifungal activity.

RESULTS AND DISCUSSION

Preliminary phytochemical screening

Ethanolic extract of *Clemantis montana* leaves showed the presence of carbohydrates, tannins, steroids, triterpenoids, saponins and flavonoids table 1. Medicinal plants are a great source of active constituents for the development of new therapeutic compounds.

Table 1: Results of preliminary phytochemical investigation *of ethanolic extracts of C. montana* leaves

S. No.	Test	Leaves
1.	Alkaloids	-
2.	Carbohydrates	+
3.	Steroids and Sterols	+
4.	Flavonoids	+++
5.	Tannins	+++
6.	Triterpenoids	+
7.	Saponin Test	+

Physicochemical parameters screening

Physicochemical parameters of *Clemantis montana* leaves has been depicted in table 2. The percentage of total ash, acid insoluble ash, water soluble ash value has been found 8.20, 1.50 and 2.28% respectively. The extractive value has been found 16.64% in water and 7.52% in alcohol. 2% foreign matter and 8.9% moisture content has been found in *Clemantis Montana* leaves. These herbal drugs were standardized as per WHO guidelines. The moisture content or loss on drying less than the limit, it means less chances of bacteria, fungi or yeast growth. Ash value of a drug gives an idea of the earthy matter or inorganic composition, and other impurities present along with the drug. Extractive values are primarily useful for the determination of exhausted or adulterated drugs. All these parameters, which are being reported, could be useful in identification of distinctiveness features of the drug and also valuable in manufacturing as raw material or in prescription medicine.

Table 2: Physicochemical parameters of *Clemantis montana*

S. No.	Physicochemical parameter	Type	Observed value (% w/w)
1.	Ash value	Total ash	8.20
		Acid insoluble ash	1.50
		Water soluble ash	2.28
2.	Extractive value	Alcohol soluble value	7.52
		Water soluble	16.64
3.	Foreign matter		2
4.	Moisture content		8.9

***In vitro* antioxidant studies**

DPPH method

The DPPH free radicals scavenging activity has been shown in table 3. The IC_{50} value was found to be 151.50 µg/ml for extract and 6.727 µg/ml for ascorbic acid. DPPH (1, 1-diphenyl-2-picryl hydrazyl radical) is commonly used to find out the anti-oxidant property of plants extract. When DPPH react with the antioxidant, it's converted into DPPHH and absorbance decreases from DPPH radical to DPPH-H form [20]. In the presence of antioxidant molecules, DPPH dark purple colour change to a colourless solution. Discoloration of DPPH solution directly proportional to antioxidant property of the sample.

Table 3: IC_{50} value of ethanolic extract of leaves of *Clementis montana* and standard ascorbic acid by DPPH method

S. No.	Extract		Ascorbic acid	
	Concentration(µg/ml)	%inhibition	Concentration(µg/ml)	%inhibition
1	100	40.97	2	13.22
2	200	58.42	4	23.42
3	300	72.56	6	43.24
4	400	80.66	8	59.04
5			10	80.22
	IC_{50}	151.50 µg/ml	IC_{50}	6.727 µg/ml

Ferrous chelating activity

In the case of the ferrous chelating method, chelating rate has been increased with the increasing concentration of ethanolic extract of *Clemantis Montana* leaves, but it is less compare to the standard BHT (table 4). In metal chelating assay, ferrozine forms a magenta colour complex with Fe^{2+}. In the presence of an antioxidant, the colour intensity of the sample decreases [21].

Table 4: IC_{50} value of ethanolic extract of leaves of *Clementis montana* and standard BHT by ferrous chelating method

S. No.	Extract		BHT	
	Concentration(µg/ml)	%inhibition	Concentration(µg/ml)	%inhibition
1	200	32.25	100	44.12
2	400	43.52	200	52.24
3	600	54.63	300	64.26
4	800	66.81	400	82.21
5	1000	72.44		
	IC_{50}	532.94 µg/ml	IC_{50}	165.63 µg/ml

Reducing power assay

IC_{50} value in reducing power assay 254.5 µg/ml for extract and43.7 µg/ml for standard (table 5). Substances, which have reduction potential, react with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form a ferric ferrous complex that has an absorption maximum at 700 nm. These assays are known as a robust and useful method for measuring a wide concentration range of antioxidant activities and capacities [22].

Table 5: IC_{50} value of ethanolic extract of leaves of *Clementis montana* and standard ascorbic acid by reducing power assay

S. No.	Extract		ascorbic acid	
	Concentration(µg/ml)	Absorbance	Concentration(µg/ml)	Absorbance
1	100	0.212	20	0.282
2	200	0.452	40	0.48
3	300	0.624	60	0.672
4	400	0.894	80	0.884
5			100	1.12
	IC_{50}	254.5µg/ml	IC_{50}	43.7µg/ml

Antimicrobial activity

Antimicrobial activity was carried out for ethanolic extract of leaves of *Clemantis montana*. The data for antibacterial and antifungal activity is listed in Table-6. From the data, it is evident that ethanolic extract of leaves of *Clemantis montana* showed the greater zone of inhibition against gram-positive than gram-negative bacteria. This shows that gram-positive bacteria are more susceptible than gram-negative bacteria. The extract also showed significant antifungal activity against *C. albicans*. The results of present study indicate that the plant extract showed both antibacterial and antifungal activity against the test organisms which might be due to the phytoconstituents present in the leaves.

Table 6: Antimicrobial activity of ethanolic extract of *Clemantis montana* leaves

Concentration	Zone of inhibition (mm) of *Clemantis Montana* leaves				
	Antibacterial activity				Antifungal
	Gram-positive		Gram-negative		
	S. aureus	*B. subtilis*	*E. coli*	*P. aeruginosa*	*C. albicans*
100 µg/ml (extract)	10	11	18	15	14
250 µg/ml (extract)	14	18	21	22	16
500 µg/ml (extract)	18	20	24	23	19
1000 µg/ml (extract)	21	24	28	25	22
Control	--	--	--	--	--
Amoxycillin10µg/ml	13	14	11	12	--
Fluconazole10µg/ml	--	--	--	--	22

CONCLUSION

As per above-mentioned results of antioxidant, it is concluded that *C. Montana* can be a potential source of antioxidant. This extract shows significant antibacterial and antifungal activity in comparison with amoxicillin and fluconazole respectively.

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CONFLICTS OF INTERESTS

We declare that we have no conflict of interest

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