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Research Article

PHYTOCHEMICAL ANALYSIS OF THE LEAVES OF *CLERODENDRUM VISCOSSUM* VENT.

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ABSTRACT

Objectives: The aim of the present study was to analyse qualitatively and quantitatively the phytochemical profile of *C. viscossum* leaves.

Methods: Various standard biochemical and spectrophotometric methods were employed to study the phytochemical status of the leaves of *C. viscossum*. All the analysis were performed in multiple sets.

Results: Results indicated the presence of various chemicals like flavonoid, glycoside, tannin, alkaloid, trepinoid, phenolics etc. The total alkaloid, flavonoid and phenolic content were found to be 1.30±0.09 g/100 g, 16.04±1.33 mg/g and 57.48±0.71 mg/g respectively besides the presence of certain amount of tannin (46.63±0.03 mg/100 g) and saponin (13.31±0.47 g/100 g) content. Among the three vitamins, total riboflavin (0.67±0.01 mg/100 g) and ascorbic acid (0.66±0.00 mg/100 g) content were found to be high, followed by thiamine content of 0.37±0.02 mg/100g.

Conclusion: From the present study it may be concluded that the leaves of *C. viscossum* contains very high amount of various phytochemicals. These phytochemicals are chiefly responsible for various medicinal properties of a plant. Therefore, presence of the phytocompounds in high quantity may lead to the potent medicinal capacity of *C. viscossum* leaves.

Keywords: Antioxidant, Anti-cancer, Clerodendrum, Flavonoid, Glycoside, Herbal Medicine, Phenol, Phytochemical, Plant Extract, Principal component analysis.

INTRODUCTION

Phytomedicine is the most primitive therapeutic approach known to man. Since the dawn of civilization men have experimented, used, relied and passed the knowledge of herbal medicines from generations to generations which resides among ethnomedicinal domain. Today, this falls under the complementary an alternative medicine (CAM). Phytotherapy continues to play a vital role in the present health care system, with around 80% of world's population still primarily relying on plant based medicine [1]. In a country like India which is not only the birthplace of Ayurveda but also holds a huge traditional, folklore and tribal knowledge of CAM, the *material medica* of these systems contain rich heritage of medicinal plants to sustain healthy life of the common people.

Certain extracts and chemicals derived from these plants have beneficial effect on the body in the form of antioxidants, hepatoprotectants, anti-cancer agents, immunostimulants and display a vast array of therapeutic value. The core of these herbal formulations consists of certain class of phytochemicals having therapeutic value of these plant materials. The ayurvedic concept of "Rasayana" i.e. medicinal compositions which enhance body resistance through promoting the physical and mental health, improving body's defence mechanisms against infections and disease [2], also talks about the essentiality of the correct phytochemical composition of herbal formulations. Therefore, it is the phytochemicals in these formulations working either synergistically or individually, playing the major role in disease resistance and treatment. With the advent of complications such as multidrug resistance, toxicity and side effects associated with the modern synthetic drugs and antibiotics [3], today the main focus of the medicinal science is on the phytochemistry of these herbal formulations. Thus, plants with high phytochemical profile holds great chance to be considered for its therapeutic value. *C. viscossum* (family Verbenaceae) is an indigenous perennial shrub of India. Various medicinal-bioactivities of this plant including antihemolytic [4], antihelmintic [5], antioxidant [6], antibacterial [7], analgesic activity [8] etc has been studied extensively. But to our knowledge, the detailed phytochemical analysis of the leaves of *C. viscossum* has not been performed yet. Therefore, the aim of the present study was to qualitatively analyse the presence/absence of various phytochemicals in the leaves of *C. viscossum.* In addition, we have also quantitatively estimated the amount of different phytochemicals present in the leaves of *C. viscossum.*

Materials and Methods

Sample collection

Fresh, full grown and disease free leaves of *C. viscossum* were collected from the field (26.71oN, 88.35oS) of University of North Bengal during the month of May, 2013. The plant was identified by Taxonomist Prof. A. P. Das of Department of Botany, University of North Bengal. The voucher specimen was stored in the Botany department herbarium with an accession number of 9617.

Sample preparation

The leaves were washed properly with tap water initially and then twice with double distilled water to remove traces dirt. The leaves were then shade dried in the laboratory for two weeks. Thereafter, the dried leaves were grinded to powder and the leaf powder was passed through a 0.5 mm metallic mesh. The resultant fine powder was packed in an airtight container for further use. Various solvent extracts were prepared using fine powder by dissolving in the suitable solvents accordingly.

Chemicals

Chemicals were obtained from Sisco Research laboratories Pvt. Ltd. (Mumbai, India), unless otherwise indicated. Analytical grade sulphuric acid, chloroform, acetic acid, ethyl acetate, trichloroacetic acid (TCA), diethyl ether and isoamyl alcohol were procured from Merck Specialties Pvt.Ltd. (Mumbai, India). α-napthol, ferric chloride, sodium sulphate, bovine serum albumin (BSA), tannic acid, gallic acid, thiamine, riboflavin and were obtained from HiMedia laboratories Pvt. Ltd. (Mumbai India). HCL was supplied by Thomas Baker (Mumbai. India).

Qualitative profiling

Qualitative assessment for the major classes of phytochemicals namely tannin, phlobatannin, terpenoid, glycoside, phenolics, flavonoids, steroids, anthraquinone, saponin, alkaloid and major biomolecules namely cholesterol (lipid), carbohydrate and protein were performed according to the various standard methods [1,9,10,11].

Quantitative profiling

Quantitative analysis of the following phytochemicals were performed according to the standard protocols.

Estimation of Riboflavin content

Standard method of Poornima *et al*., [12] was followed to measure the total riboflavin content in the leaves. Briefly, 10 g of crude powder was taken in a 250 ml conical flask and 100 ml 50% ethanol was added to it. The mixture was stirred for 4 h on a magnetic stirrer at room temperature. The solution was filtered and 10 ml of 5% potassium permanganate solution was added to it. The mixture was stirred using a glass rod continuously and 10 ml of 30% hydrogen peroxide was added to it simultaneously. This was allowed to stand over a 80 ^{o}C water bath for 30 min. Two ml of 40% sodium sulphate was added to it and the absorbance was measured at 510 nm using Rayleigh UV-2601 spectrophotometer. A blank was prepared with all the chemicals except the plant material.

Quantification of total Ascorbic Acid content

The previously standardised method [13] was followed to estimate the ascorbic content in the leaves. Five grams of powder sample was mixed with 100 ml extraction mixture (trichloroacetic acid/etheline diamine tetra acetic acid at 2:1) and stirred on a magnetic stirrer for 3 h at room temperature. This was transferred to a centrifuge tube and centrifuged at 2000 rpm for 30 min. After centrifugation the supernatant liquid was filtered. 2-3 drops of 1% starch indicator was added to the filtrate and it was titrated against 20% $CuSO_4$ solution until a dark end point is reached.

Estimation of Thiamine content

Quantification of thiamine was done according to the spectrophotometric method of Nahapetian *et al*. [14]. Fifty grams of plant sample was dispersed in 50 ml ethanolic sodium hydroxide (20%) and stirred over a magnetic stirrer for 3 hours at room temperature. The resultant was filtered into a 100 ml conical flask. Ten ml of the filtrate was separated out and 10 ml of 2% potassium dichromate solution as added to it. As a result a colour was developed and then the solution was read at 360 nm against a suitable blank. The blank contained all except the plant material.

Determination of total Saponin content

Total saponin content was estimated according to the method of Nahapetian and Bassiri [14]. Briefly, 10 g of sample powder was taken in a flask and 100 ml of 20% ethanol was added to it. The mixture was heated over a hot water bath at 55 ^{o}C for a period of 5 h with continuous stirring. The mixture was filtered and the supernatant liquid was separated. The solid residue was mixed with another 100 ml of 20% ethanol and stirred for 5 h as done previously. The solution was filtered and the extract was mixed with the previously stored extract. The combined extract was placed on a hot water bath of 90^{o} C and heated till the volume of the extract was reduced to 1/5th of its initial volume. The concentrate was transferred into a 250 ml separating funnel and 10 ml of diethyl ether was added to it and shakened vigorously. After the solution settles down, the aqueous layer was separated carefully into another flask and the ether layer was discarded. The purification process was repeated again. 60 ml of n-butanol extracts were washed twice with 10 ml of 5% aqueous NaCl solution. The remaining solution was heated in a water bath at 50^{o} C until the solvent evaporates and the solution turns to semi dried form. The sample was then dried in an oven into a constant weight. The saponin content was calculated by the following equation:

Percentage of saponin = (W_{EP} / W_S) X 100

Where, W_{EP} = Weight of oven dried end product.

W_S = Weight of powdered sample taken for test.

Quantification of total Alkaloid

Total alkaloid content was determined according to the method of Obadoni *et al*., [15].

Five grams of sample powder was taken into a 250 ml beaker and 250 ml of 20% CH_3COOH in ethanol was added into it. The mixture was shaken on a magnetic stirrer for 10 h at room temperature. The solution was filtered through Whatman filter paper no. 1 and the resultant was placed on a hot water bath (60^{o}C) until the extract volume turns 1/4th of its initial volume. Concentrated NH_4OH was added drop wise which forms thick precipitate. NH_4OH was added until the precipitate formation was completed. The whole solution was allowed to settle down. The precipitate was collected by filtration, dried in an oven and weighed.

Estimation of total Flavonoid content

Standard method of Boham *et al*., [16] was followed with slight modifications to quantify the total flavonoid. Ten grams of plant powder was taken in a flask and 100 ml of 70% methanol was added. The mixture was stirred using a magnetic stirrer for 3 h and filtered through Whatman filter paper number 1. The remaining powdered material was re-extracted once again with 70% methanol and filtered in a similar way. Both the filtrates were mixed and transferred into a crucible and evaporated to dryness over a water bath of 60^{o}C and weighed.

Determination of total Tannin content

A previously described standard method was followed to estimate the total tannin content in the leaves of *C. viscossum* [17]. Briefly, 1 g of crude powder sample was taken in a 100 ml flask and 50 ml of double distilled water was added and shook on a magnetic stirrer for 10 h at room temperature. It was filtered into a 50 ml volumetric flask and made up to the mark using distilled water. Five ml of solution was pipetted out in a test tube and 0.008 M $K_4[Fe(CN)_6]$ and 0.1 M $FeCl_3$ in 0.1 N HCl were added to it. The absorbance was measured in spectrophotometer at 120 nm wavelength within 10 min. A blank was prepared and read at the same wavelength. A standard was prepared using tannic acid to get 100 ppm and measured.

Estimation of total poly phenolic content

The test sample needed to be fat free to estimate total phenol [15] content. To prepare fat free sample, 5 g of crude plant powder was mixed with 100 ml n-hexane and defatted using a soxlet apparatus for 2 h. The resultant was used for the determination of total phenol.The fat free sample was then boiled with a 50 ml of ether for 15 min for the extraction of total phenolic content. The resultant was filtered and 5 ml of the filtrate was pipetted out in 50 ml flask. Ten ml of double distilled water was added to it.

Two ml of NH_4OH solution and 5 ml of concentrated amyl alcohol were added to the solution with constant stirring. The flask was incubated at room temperature for 30 min for the development of colour. The absorbance of the solution was read using spectrophotometer at 550 nm against a suitable blank. The phenolic content was evaluated from a gallic acid standard curve.

Total protein content determination

Total protein was estimated according to the method proposed by Lowry *et al.* [18] with slight modifications. Known concentrations of bovine serum albumin was taken as standard and the OD was read at 750 nm using a suitable blank.

Quantification of total lipid content

The assay was performed according to a standard method [19] with slight modifications. One gram of dried sample was macerated with 10 ml distilled water. Thirty ml of chloroform-methanol (2:1 v/v) was mixed thoroughly to it and the mixture was left for overnight at room temperature.

Twenty ml of chloroform and equal volume of distilled water was added and centrifuged at 1000 rpm for 10 min. After centrifugation three layers were formed, out of which the lower layer was collected which contained chloroform containing lipid. The mixture was kept in an oven for one hour at 50 ^{o}C fir the evaporation of chloroform. Weight of the residue was measured.

Total Sugar content Estimation

Total sugar content was determined according to Dubois *et al*., [20] with slight modifications. Fifty grams of the powdered test sample macerated in a pestle and mortar with 20 ml of ethanol and kept for incubation at 30 ^{o}C for 10 h. The mixture was centrifuged at 1500 rpm for 20 min and the supernatant was collected separately. To 1 ml of the alcoholic extract, 1 ml of 5% phenol solution was added and mixed. Five ml of concentrated H_2SO_4 was added rapidly with constant stirring. This was allowed to stand for 30 min at room temperature. The colour of the solution changed in to yellow orange and the OD was measured at 490 nm against a blank. The blank standard curve was prepared using known concentrations of glucose. The quantity of the sugar was expressed as mg/g fresh weight of the sample.

Determination of Moisture and Ash Content

Moisture and ash content of different parts of the plant was estimated by subjecting specific amount of sample to 90 ^{o}C for 12 h in an oven and at 400 - 450^{o} C in a furnace for 5 min respectively. The resultant weight was calculated for moisture and ash content estimation respectively.

Statically analysis

All the experiments were performed in triplicates and the data were reported as the mean ± SD of the three measurements. Statistical analysis was performed using KyPlot version 2.0 beta 15 (32 bit). A Principal Component Analysis (PCA) based on the correlation matrix was performed using the SPSS statistics version 20.0 software package to find out any possible interrelation among the phytochemicals quantified.

RESULTS

Observations of the qualitative tests are enlisted in table 1.

Table 1: Displays the presence/ absence of different phytochemicals in the leaves of *C. viscossum*

Phytochemical Parameters	Presence
Tannin	+
Phlobatannin	+
Cholesterol	+
Terpinoid	+
Glycoside	+
Phenolics	+
Flavonoid	+
Steroid	+
Anthraquinone	+
Saponin	-
Carbohydrate	+
Protein	+
Alkaloid	+

The results of the quantification of phytochemicals in the leaves of *C. viscossum* are enlisted in table 2.

Table 2: Describes the results of the descriptive statistics for the thirteen parameters studied namely flavonoid, alkaloid, saponin, phenol, ascorbic acid, thiamine, riboflavin, total protein, lipid, soluble sugar, tannin, moisture and ash content. S.D. = Standard deviation; SEM=Standard error of mean; Coef.Vr=Co-efficient of Variance. All values are the mean of three replicate experiments. # Units are in g/100 g; $ Units are in mg/g; @ Units are in mg/100 g; δ Units are in %.

	Sum	Mean	S.E.M.	S.D.	Variance	Coef. Var.
Tannin@	139.91	46.63	1.51	0.03	6.87	0.05
Alkaloid#	3.924	1.30	0.05	0.09	0.00	0.06
Saponin#	39.93	13.31	0.27	0.47	0.22	0.03
Phenol$	172.45	57.48	0.41	0.71	0.51	0.01
Flavonoid$	48.14	16.04	0.77	1.33	1.79	0.08
Ascorbic acid@	1.99	0.66	0.00	0.00	3.33	0.00
Thianine@	1.12	0.37	0.01	0.02	0.00	0.05
Riboflavin@	2.03	0.67	0.00	0.01	0.00	0.01
Protein$	5.73	1.91	0.34	0.60	0.36	0.31
Lipid$	86.74	28.91	0.13	0.23	0.05	0.00
Sugar$	1.62	0.54	0.012	0.02	0.00	0.04
Moisture$	181.53	60.51	0.35	0.61	0.37	0.01
Ash$	11.37	3.79	0.10	0.61	0.03	0.04

Table 3: Represents the correlation matrix of different phytochemicals based on the Principal Component Analysis performed by SPSS statistics version 20.0 software package. Where, *Correlation is significant at the 0.05 level (1-tailed).**

	Tannin	Alkaloid	Saponin	Phenol	Flavonoid	Ascorbic	Thianine	Riboflavin	Protein	Lipid	Sugar	Moisture	Ash
Tannin	1												
Alkaloid	0.894	1											
Saponin	0.384	-0.07	1										
Phenol	0.984	0.799	0.543	1									
Flavonoid	-0.959	-0.984	-0.107	-0.893	1								
Ascorbic	0.995*	0.933	0.294	0.962	-0.982	1							
Thianine	0.184	0.605	-0.837	0.006	-0.455	0.277	1						
Riboflavin	-0.58	-0.154	-0.975	-0.717	0.326	-0.5	0.693	1					
Protein	-0.808	-0.458	-0.855	-0.9	0.607	-0.748	0.431	0.949	1				
Lipid	-0.888	-0.587	-0.766	-0.956	0.721	-0.84	0.289	0.89	0.988*	1			
Sugar	0.996*	0.848	0.47	0.996*	-0.928	0.982	0.091	-0.655	-0.86	-0.92	1		
Moisture	-0.531	-0.854	0.579	-0.37	0.749	-0.609	-0.931	-0.382	-0.071	0.081	-0.44	1	
Ash	-0.241	0.22	0.989*	-0.41	-0.044	-0.147	0.91	0.93	0.767	0.66	-0.33	-0.695	1

The correlation matrix (table 3) display inter-relations between the various phytochemical parameters. The highest positive correlation (0.99) occur between tannin with sugar and ascorbic acid content ($p<0.05$; 1-tailed). Similar high correlation resided between sugar content with total phenol ($p<0.05$; 1-tailed). Highest negative correlation (-0.07) resided between saponin with alkaloid and protein with moisture content ($p<0.05$; 1-tailed).

Multivariate statistical analysis and correlation patterns

Principal component analysis (PCA) is a dimension reduction technique under the multivariate statistical approach which allows to visualize the underlying pattern of the variables in an experiment [21]. In PCA, the variables are cumulatively arranged in the most simplified way to highlight the overall picture of the correlation between them. Presently, PCA is used to analyse data sets of different branches of the biological science ranging from study of correlation pattern of cytokines [22], global genetic structure analysis [23], study of antioxidant profile of therapeutics [21], microarray data analysis [24] etc. Therefore, in the present investigation, PCA was performed to understand how the thirteen parameters namely flavonoid, alkaloid, saponin, phenol, ascorbic acid, thiamine, riboflavin, total protein, lipid, soluble sugar, tannin, moisture and ash content contribute to the overall phytochemical profile of the *C. viscossum* leaf extract. The loading plot (fig. 1) of first and second principal components, (PC1 and PC2) accounted for 62.91% and 37.08% of variance, respectively. Alkaloid, ascorbic acid, tannin, sugar and phenol content was loaded high on PC1 making a separate cluster from the other variables, a with loading value of 0.952, 0.998, 0.988, 0.970, 0.945, respectively. Riboflavin, ash and thiamine content were highly loaded on PC2 with loading value of 0.893, 0.996, 0.943, respectively. Besides, flavonoid content resided very high negative on PC1 (-0.991) and saponin content was loaded highly negative on PC2 (-0.971).

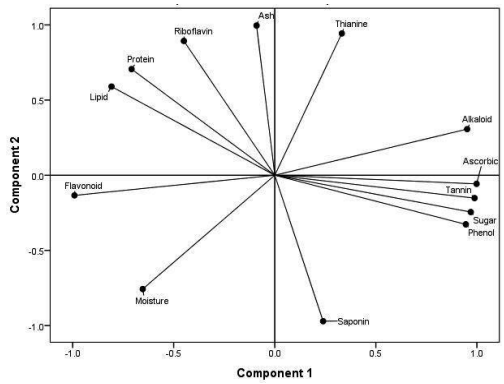

Fig 1: Represents the principal component analysis (PCA) under varimax rotation for the phytochemicals of *C. viscossum.*

DISCUSSION

Phytochemicals are the core of phytomedicin. The therapeutic efficiency of phytomedicines directly correlates with the presence of various phytochemicals. In this study, we have identified and quantified the major phytochemicals present in the leaves of *C. viscossum*. Flavonoids are one of the major classes of phytochemicals associated with diverse pharmacological activities which include antioxidant, anti-cancer and anti-aging properties [25]. Flavonoid belong to the largest group of phenolics, consisting more than 600 natural compounds [26]. Generally, flavonoids with two 6-carbon rings and one 3-carbon linkage (usually forming a 3rd ring) are divided into chalcones, flavones, flavonols, flavanones, isoflavones, flavan-3-ols, and anthocyanins, according to the modification of the basic carbon skeleton [26, 27]. Evidences suggest that flavonoids may bind to gamma-aminobutyric acid A receptors having sedative or anxiolytic effects on the nervous system and also can act to up-regulate the cholinergic nervous system [26, 28]. Flavonoid-rich supplements may reduce neurodegenerative processes and abnormal detoriations in cognitive performance [29]. Alkaloids are nitrogen based natural compounds, majority of which belong to plant secondary metabolites. Till date over 12,000 diverse alkaloids were identified from one quarter of the plant species [30]. The alkaloid morphine from the plant *Papaver somniferum* is extensively used to relieve from severe pain. Furthermore, in modern medicine Artopine is used as an antidote to nerve-gas poisoning, caffeine is a central nervous system stimulant, Sanguinarine is antibacterial, vincristine and vinblastine are used as anti-cancer agents, reserpine as anti-hypersensitive and quinine as anti-malarial agents. Saponins are also plant secondary metabolite characterized by its ability of foaming in aqueous solution. They have a wide range of industrial application ranging from serving as raw material for steroid hormone synthesis, use as food additive, in fire extinguisher etc [31, 32]. Saponins also exhibit a wide range of biological activity such as immunostimulatory, anti-inflammatory, hypocholesterolemic activity etc [33].

Tannins has been considered in traditional medicine to treat various diseases and included in several pharmacopoeias referring as "acidum tannicum" or tannic. Regression of viral activities has been found to be associated with high tannin content [34]. Synergistic effects of certain tannins with various antibiotics have been found beneficial against antibiotic resistance bacteria [35]. Moreover, anti-cancer, anti-mutagenic and tumor promotion inhibitory activity have also been found to be associated with tannins [35].

A large variety of plant metabolites possessing potent bioactivity belong to a group of phenolic compound and their derivatives. Antioxidant and reactive oxygen species (ROS) scavenging capacity of plant is primarily attributed the presence of phenolic compounds. Various phenolic compounds hold the ability to inhibit the formation of ROS by inhibiting activation of redox sensitive transcription factor like nuclear-factor κβ [36].

Various plant derived phenolic compounds such as curcumin, genistein, resveratrol and catechins act as potent inhibitors of growth factor and signalling pathways associated with cancer [36]. Resveratrol has the ability to halt cell-cycle at various stages in different cancer models [36]. Plant derived phenolics have also demonstrated immunomodulatory activity by modulating cytokines and chemokines [37, 38].

On the other hand vitamins not only act as micronutrients but are also as one of the essential components for the prevention of diseases. Riboflavin is essential for normal growth and development of our body and is responsible for release of energy during carbohydrate metabolism. Ascorbic acid is predominantly functions as antioxidant and ROS scavenger and is essential for growth and repair of tissue. Thiamine plays a major role in the electrolyte balance of muscle and nerve cells.

Therefore, phytochemicals play a vital role in prevention of diseases. Moreover, out of 250,000 higher plant species, more than 70,000 species are utilized as therapeutic in different ethnomedicinal practices [39]. The medicinal property [4,5,6,7,8] of *C. viscossum* may be due to the presence of the phytochemicals.

CONCLUSION

Different plants of the genus *Clerodendrum* has been extensively studied for their medicinal properties and also have their names mentioned in different traditional medicinal practices [6]. Some of the plants are also mentioned in various traditional literatures. However, *C. viscossum* is one of the least studied plant of this genus. PubMed search with the keyword *Clerodendrum viscosum* resulted in only 5 hits.

In the present investigation, we have studied the preliminary phytochemical profile of the plant. Bio-induction studied may prove beneficial for improving yield of these metabolites. Detailed phytochemical analysis for the presence of bioactive compounds will be performed using HPLC and GC-MS in the future.

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