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Chandana Satheesh Kumar TP
M.Sc. Chemistry Scholar, St Marys College, Sulthan Bathery, Wayanad, Kerala, India

Nisha AR
Assistant Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

Corresponding Author:
Chandana Satheesh Kumar TP
M.Sc. Chemistry Scholar, St Marys College, Sulthan Bathery, Wayanad, Kerala, India

Proximate and phytochemical analysis of methanolic extract of *Cissus quadrangularis*

Chandana Satheesh Kumar TP and Nisha AR

Abstract
The present work was conducted to evaluate the proximate contents, phytochemical constituents and to screen the methanolic extract of the stem of *Cissus quadrangularis* for the presence of active compounds. Methanol was the solvent used for the soxhlet extraction. The dry matter content and the total ash, crude protein, crude fat, crude fibre, acid insoluble ash, carbohydrate content were determined. The chemical composition of methanolic extract of *Cissus quadrangularis* revealed the presence of alkaloids,, saponins, phytosterols, phenolic compounds, flavanoids, terpenoids, protein, amino acids, fixed oils, fats, tannins and cardiac glycosides. This work could be further useful to the structural elucidation and further quantification of bioactive compounds.

Keywords: *Cissus quadrangularis*, methanolic extract, proximate analysis, phytoconstituents

1. Introduction
Plant based products are healthier, safer and reliable than the synthetic products. Nowadays drug industries depend on plant molecules for new drugs because of the side effects of synthetic products. Approximately 80% of the people depend on traditional medicines for their primary health care according to WHO (Suhasini *et al.*, 2015) [7]. Plant constituents comprise a wide variety of organic substances namely primary metabolites and secondary metabolites. Alkaloids, phenolics, lignins, essential oils, steroids, tannins etc. belongs to the category of secondary metabolites. Secondary metabolites are also classified into nitrogen containing compounds, terpenoids, phenolics (Wink, 1987) [10]. *Cissus quadrangularis* (*C. quadrangularis*) belongs to Vitacea family is a common perennial climber which is distributed throughout in India particularly in hotter parts (Shukla, 2015) [6]. Determination of flavanoids is very important because of pharmacological effects (Tamanna *et al*., 2017) [8]. Therefore, the present investigation was aimed to Evaluate the proximate contents, phytochemical constituents of methanolic extract of the stem of *C. quadrangularis*.

2. Materials and Methods
2.1. Chemicals
All the chemicals and reagents used in this study were procured from M/S Merck India, Ltd., India / Germany.

2.2 Plant Materials
2.2.1 Collection and identification of plant materials
The taxonomically identified *C. quadrangularis* plant stem used in this study were collected during April, 2017 from Karnataka. The plant was identified and authenticated by a botanist in the Department of botany, Calicut University. A herbarium for morphological studies was prepared and a voucher specimen deposited at Calicut University herbarium (Accession No.148208), Kozhikode, Kerala.

2.2.2. Preparation of plant materials
Plant materials collected were cleaned and dried under shade to remove water. They dried stems were powdered in an electrically driven mortar and sieved to remove the coarse particles. The fine powder obtained was stored in airtight container until use.

2.2.3. Preparation of crude methanolic extract
The powdered plant material of *C. quadrangularis* was used for extraction using methanol in

soxhlet extraction apparatus attached with a rotary vacuum evaporator (Buchi, Switzerland). Solvents were removed using rotary vacuum evaporator at 96 mbar at a temperature in the range of 40 ^{0}C to 45 ^{0}C. The weight of the dried extract was recorded and the extractive yield was calculated as,

$$\text{Extractive value} = \frac{\text{Weight of the extract} \quad X100}{\text{Weight of powered plant sample taken}}$$

Crude extracts were subjected to phytochemical analysis by dissolving the extract in methanol.

Fig 1: Whole plant *Cissus quadrangularis* in its natural habitat

2.3 Analysis of Plant Material

2.3.1. Proximate analysis of the potent crude drug powder

The proximate analysis of ash, crude fibre, crude protein, carbohydrate, crude fat, dry matter and moisture content of respective plants were carried out by using standard proximate analysis techniques as mentioned by Pathak, 1996 [5].

2.3.1.1. Determination of dry matter

The dry matter was determined using the weight difference method and estimated by deducting per cent moisture from hundred as described by James (1995) [4].

Dry matter (per cent) = 100 – per cent of moisture

The moisture content was determined by using the formula,

$$\text{Per cent of moisture content} = \frac{\text{Weight of sample} - \text{weight of dried sample X 100}}{\text{Weight of sample}}$$

2.3.1.2 Determination of total ash contents

Ash represents the inorganic content of the sample which is determined by the method as described by Association of Official Analytical Chemists (AOAC, 2000). Total ash content was calculated by the following formula,

$$\text{Ash (per cent)} = \frac{\text{Weight of ashed sample} \times 100}{\text{Weight of sample taken}}$$

2.3.1.3. Determination of crude fat

One gram of crushed dried sample was taken in the filter paper thimble kept in a pre-weighed flask of fat extractor. Petroleum ether (80 ml) was poured into the flask and refluxed for 8 hours. The flask was cooled in desiccator and the weight of crude fat so extracted was taken. The per cent crude fat was determined by using formula,

$$\text{Crude fat (per cent)} = \frac{\text{Weight of flask with fat - weight of empty flask x 100}}{\text{Weight of original sample}}$$

2.3.1.4. Determination of crude fiber

One gram of the defattened plant material was taken in a spoutless beaker and boiled in 200 ml of 1.25 per cent sulphuric acid for 30 minutes. The content was then filtered, washed with hot distilled water to neutralize, transferred again to the beaker and boiled in 200 ml of 1.25 per cent sodium hydroxide for 30 minutes followed by filtration and washing with hot distilled water for neutralization. The crucible was then dried in a hot air oven at 100 ± 5 ^{0}C overnight (10-12 hr), cooled in a desiccator and weighed to a constant weight. Subsequently, the desiccator with its content was put in a muffle furnace at 550-600 ^{0}C for 2-3 hour for complete burning of organic matter and cooled in a desiccator and weighed to a constant weight. The per cent of crude fibre was determined from the formula,

$$\text{Crude fibre (per cent)} = \frac{(W_1 - W_2) \quad x\ 100}{\text{Weight of sample}}$$

Where,

w_1- The crucible having crude fibre was cooled and weighed

w_2- The content of the crucible was ignited over a low flame until charred and then kept in a muffle furnace and weighed.

2.3.1.5. Determination of crude protein

Total nitrogen (N) content was determined with the help of Kjeldahl method described by Pearson (1976).

The per cent of protein was calculated by the formula.

$$\text{Protein (per cent)} = \frac{V \times 1.4 \text{ x } 6.25 \text{ x } 0.1N \text{ HCl x Vol (used)} \times 100}{W \times A \times 1000}$$

Where;

V= Titre value

1.4 = Weight of nitrogen expressed in gram in the formula

6.25 = Protein factor

W= Weight of sample

A= Aliquot digested sample used for distillation

d) Determination of carbohydrate

Determination of available carbohydrate in the sample was calculated from the above parameters by using the formula.

Carbohydrate (per cent) = 100 - (moisture + crude fat + ash + protein) per cent

2.3.1.6. Determination of calcium

The acid soluble mineral extract obtained in acid insoluble ash experiment is used for estimation of calcium and phosphorus. Ten ml of the soluble mineral extract was pipetted out into a beaker of 250ml capacity and 90 ml distilled water was added to increase the volume. The pH was adjusted to between 2.5 to 3 before the addition of the precipitating reagent *viz.* saturated ammonium oxalate.

Precipitation of calcium: After adjusting the pH, the beaker with its content was placed over the hot plate. The contents of the beaker was gently heated and then 10 to 15ml of warm

ammonium oxalate (precipitant)was added which resulted in the precipitation of calcium as calcium oxalate (CaC_2O_4) and was seen as white granular precipitate.
Barium chloride test: Barium chloride (5%) solution was used to test whether the precipitate was free from oxalate. The appearance of white precipitate between the barium chloride and filtrate solution indicated the presence of oxalate which necessitated further washing.
Titration: 0.1N $KMnO_4$was used in this titration. The content of the beaker was heated till few bubbles appeared.
1 ml of 0.1N $KMnO_4$ = 0.002 g of calcium or 0.0028g of calcium oxide
Calculation

$$\text{Percentage of calcium} = \frac{0.002 \times D \times B \times 100}{A \times C}$$

Where,
A = weight of substance
B = total volume of soluble mineral extract
C =aliquot taken
D = burette reading

2.3.1.7. Determination of phosphorus
Principle
The phosphorus was precipitated as yellow colour phosphor-ammonium-molybdate, by the addition of ammonium molybdate reagent. The precipitate was washed and dissolved in measured volume of standard alkali. The amount of alkali, which has dissolved the phospho-ammonium- molybdate precipitate, is indirectly found out by back titrating with standard 0.1N sulphuric acid.

$$\text{Calculation: \% of phosphorus} = 0.0001347 \times \text{BTV} \times \frac{B}{C} \times \frac{100}{A}$$

Where
A=Weight of sample
B=volume of soluble ash
C= Amount of aliquot taken
TV= Titration value
BTV= Back titration value =amount of NaOH added-TV

2.3.2. Phytochemical analysis of the crude methanolic stem extract
The phytochemical analysis of the methanolic stem extract was evaluated as per the procedure (Harborne, 1991) [3] described below.

2.3.2.1. Detection of alkaloids
The presence of alkaloids was detected by conducting the following tests. The extract was dissolved individually in dilute hydrochloric acid and filtered. The filtrate was further tested with following reagents for the presence of alkaloids.

a) Dragendroff's test: Filtrate was treated with potassium bismuth iodide solution (Dragendroff's reagent). Formation of an orange-yellow precipitate indicated the presence of alkaloids.
b) Hager's test: Filtrate was treated with saturated aqueous solution of picric acid (Hager's reagent). Presence of alkaloids was confirmed by the formation of yellow coloured precipitate.
c) Mayer's test: Filtrate was treated with potassium mercuric iodide solution (Mayer's reagent).Formation of a whitish yellow or cream coloured precipitate indicated the presence of alkaloids.
d) Wagner's test: Filtrate was treated with iodine in potassium iodide solution(Wagner's reagent). Formation of reddish brown precipitate indicated the presence of alkaloids.

2.3.2.2. Detection of carbohydrates
The presence of carbohydrates can be detected by conducting Molisch's test.

2.3.2.3. Detection of reducing sugars
The presence of reducing sugars was detected by conducting the following tests.

a) Benedict's test: The filtrate was treated with Benedict's reagent and boiled in a thermostatic water bath for five minutes. Formation of an orange red precipitate indicated the presence of reducing sugars.
b) Fehling's test: The filtrate was acidified with dilute hydrochloric acid, neutralized with alkali and heated with Fehling's solution A and B. Formation of red precipitate indicated the presence of reducing sugars.

2.3.2.4. Detection of saponins
The presence of saponins was detected by conducting the following tests.

a) Froth test: The methanolic stem extract was diluted with distilled water to 20 ml and shaken in a graduated test tube for 15 minutes. Formation of 1 cm layer of foam indicated the presence of saponins.
b) Foam test: A small quantity of the extract was shaken with 2 ml of water. Persistence of foam produced for ten minutes indicated the presence of saponins

2.3.2.5. Detection of phytosterols
A small quantity of extract dissolved in 5 ml of chloroform and subjected to Salkowski's and Libermann Buchard's tests for detection of phytosterols.

a) Salkowski's test: A few drops of concentrated sulphuric acid was added and allowed the solution to stand for some time. Formation of brown ring indicated the presence of phytosterols.
b) Libermann Burchard's test: The extract was dissolved in chloroform and chloroform extract solution was treated with a few drops of acetic anhydride, boiled and cooled. Then concentrated sulphuric acid was added. Formation of a bluish green colour solution confirmed the presence of phytosterols.

2.3.2.6. Detection of phenolic compounds
The presence of phenolic compounds was detected by conducting the following tests.
a) Ferric chloride test

2.3.2.7. Detection of tannins
Approximately 0.5 gram of the extract was boiled in 20 ml of water in a test tube and then filtered. Then a few drops of 0.1% ferric chloride was added. Development of a brownish green or a blue-black colouration indicated the presence of tannins.

2.3.2.8. Detection of flavanoids

The presence of flavanoids was detected by conducting the following tests.

a) **Alkaline reagent tests:** The methanolic stem extract was treated with a few drops of sodium hydroxide solution. Formation of an intense yellow colour, which becomes colourless on further addition of dilute acid, indicated the presence of flavanoids.

b) **Lead acetate test:** The methanolic stem extract was treated with a few drops of lead acetate solution. Formation of yellow precipitate indicated the presence of flavanoids.

c) **Ferric chloride test:** A few drops of ferric chloride solution was added to the methanolic stem extract. The development of intense green colour indicated the presence of flavonoids.

2.3.2.9. Detection of proteins and amino acids

The methanolic extract solution (100 mg in 10 ml of distilled water) was filtered through Whatman no.1 filter paper. The filtrate was tested for the presence of proteins and amino acids by conducting the following tests.

a) **Millon's test:** The test solution was treated with a few drops of Millon's reagent.A white precipitate was formed which when warmed changed to a brick red or disappeared.

b) **Biuret test:** The test solution was treated with a few drops of 2% copper sulphate solution and then 1 ml ethanol was added followed by excess of potassium hydroxide pellets. Formation of pink colour in the ethanolic layer indicated the presence of proteins.

c) **Ninhydrin test:** Ninhydrin reagent was added to the test solution and boiled for a few minutes. Formation of blue colour indicated the presence of amino acids.

2.3.2.10. Detection of terpenoids (Salkowski test)

The methanolic extract was mixed with 2 ml of chloroform and then concentrated sulphuric acid (3 ml) was carefully added to form a layer. A reddish brown coloration of the interface indicated the presence of terpenoids.

2.3.2.11 Detection of cardiac glycosides (keller-killani test)

The methanolic extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. Then 1 ml of concentrated sulphuric acid was added. Appearance of brown ring at the interface indicated the deoxy sugar characteristics of cardenolides. Appearance of a violet ring below the brown ring and a greenish ring in the acetic acid layer confirmed the results.

2.3.2.12 Test for fixed oils and fats-spot test

A small quantity of the extract was placed between two filter papers. The oil stain produced with the extract if any showed the presence of fixed oils and fats in the extracts.

2.3.2.13 Test for gums and mucilages

A small quantity of the methanolic extract was diluted with water and to it ruthenium red solution was added. Generation of a pink colour showed the presence of gums and mucilages.

3. Results and Discussion

3.1 Plant Materials

3.1.1 Collection and identification of plant materials

The collected plant part (stem) of *Cissus quadrangularis* was identified and authenticated by a botanist in the department of Botany, Calicut University. A voucher specimen of herbarium No.148208) was deposited at Calicut University Herbarium, Kozhikode, Kerala (Fig. 2).

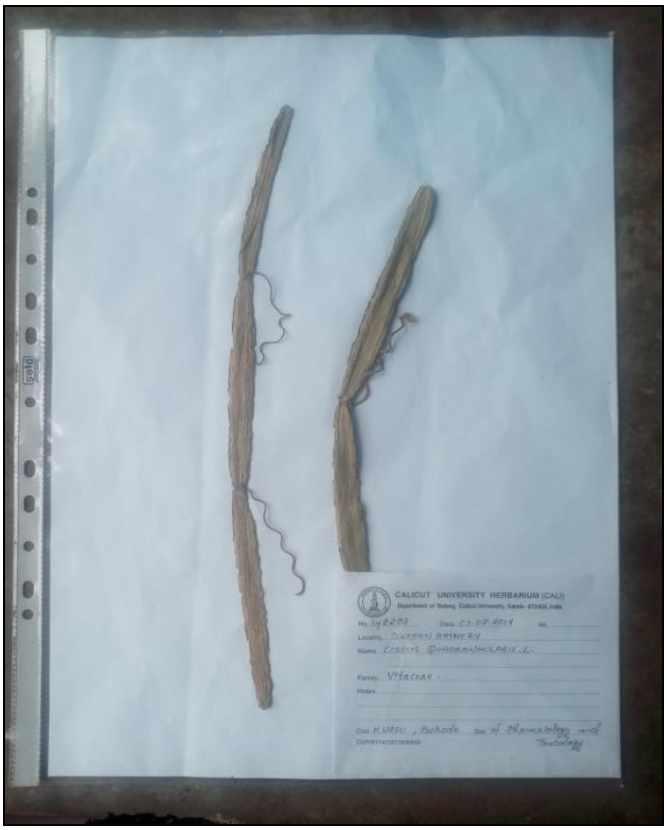

Fig 2: Herbarium of *Cissus quadrangularis* stem (No.148208)

3.1.2 Extractive values of the crude methanolic extract

The extractive values of the methanolic extracts of stem of C. *quadrangularis* was 7.09per cent.

3.2 Analysis of Plant Material

3.2.1 Proximate analysis of crude powder of stem of *C. quadrangularis*

The proximate analysis of crude powder of the stem of *C. quadrangularis* was investigated and the per cent values obtained for crude protein, crude fat, dry matter, moisture, crude fibre, ash and carbohydrate contents are shown in table 1 and figure 4.The per cent values obtained for inorganic contents of crude powder of the stem of *C. quadrangularis* is presented in Table 2. The analysis of dry matter, moisture, crude fibre, crude protein, ash carbohydrate and mineral contents of the stem of *C. quadrangularis* were carried out using standard proximate analysis techniques. The results obtained from the analysis are dry matter content was 90.75%,the total ash, crude protein, crude fat, crude fibre, acid insoluble ash and carbohydrate content were 19.39, 25.64, 3.33, 22.12, 14.34 and 42.65 per cent respectively. The percentage of calcium and phosphorus were 20.996% and 0.2416% respectively. Anuj *et al.* (2011) had detected the per cent of the total ash and acid insoluble ash value as 12.5% and 2.5% respectively. Krunal *et al.*, 2011 had observed the abundance of calcium oxalate. There are no further reports to support the findings.

Table 1: Biochemical contents in the crude powder stem of *C. quadrangularis*

Plant	Biochemical contents (%)						
	Dry Matter	Moisture	Crude fibre	Crude protein	Crude fat	Ash	Carbohydrate
Cissus quadrangularis	90.75	9.24	22.12	25.64	3.33	19.39	42.66

Table 2: Inorganic contents in the crude powder stem of *C. quadrangularis*

Plant	Biochemical contents (%)			
	Total Ash	Acid insoluble ash	calcium	Phosphorus
Cissus quadrangularis	19.39	14.34	20.99	0.24

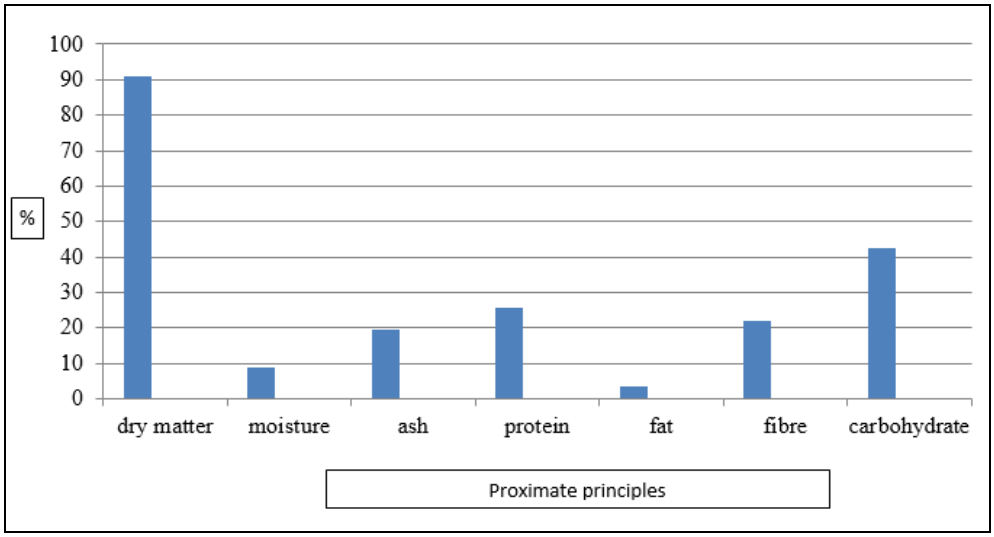

Fig 3: Figure depicting the biochemical contents in the crude powder stem of *C. Quadrangularis*

3.2.2 Phytochemical analysis of the crude methanolic stem extract

The phytochemical characteristics of methanolic extract of *C quadrangularis* investigated are summarised in table 3. The methanolic extract indicated the presence of alkaloid, saponins, phytosterols, phenolic compounds, flavanoids, terpenoids, protein, amino acids, fixed oils, fats, tannins and cardiac glycosides. The stem extract however did not reveal the presence of carbohydrates, reducing sugars, gums and mucilages. Chidambaramurthy *et al.*, (2003) [2] had detected the presence of phytosterols and tannins in the extract of *C. quadrangularis*. In another study performed by Anitha *et al.* (2014) [1] four chemical constituents were analysed from the aqueous petroleum ether and dichloromethane extract of *C. quadrangularis*, namely, alkaloids, phenol, steroids and flavanoids. Phytochemical analysis gave a significantly high presence of saponins and phytosterols. Phytosterols are a kind of plant metabolite belonging to the triterpene family. These compounds are essential biomolecules for human health, and so they must be taken along with foods. β-Sitosterol, campesterol, and stigmasterol are the main phytosterols found in plants. Phytosterols have beneficial effects on human health since they are able to reduce plasma cholesterol levels and have anti-inflammatory, anti-diabetic, and anticancer activities. Triterpenoid and steroidal glycosides, referred to collectively as saponins, are bioactive compounds present naturally in many plants. Kaur *et al*., 2015 [7] reported the presence of carbohydrates, saponins, flavanoids, alkaloids, glycosides and phenols in powdered stem material of *C. quadrangularis* which indicate its high medicinal property. The chemical constituents of *C. quadrangularis* posses novel flavanoids and indanes as well as phytosterols and keto-steroids which are powerful and efficient antioxidants. They have the potential to inhibit lipase and amylase and thereby providing a mechanism for weight loss through reduced oxidative stress, dietary fat and carbohydrate blocking.

Various pharmacological activities such as bone fracture, healing and tissue repair, prevention of osteoporosis, weight loss, blood sugar regulation, digestive disorders, menstrual irregularity, cholesterol and triglyceride lowering, antimicrobial, analgesic, antipyretic, anti-inflammatory, tissue protective, joint health have been reported and studied by many scientists (Tan Nie *et al.*, 2015) [9]. Flavanoid containing plants have many therapeutic properties like antimicrobial activity, anti-oxidant activity, free radicals scavenging activity.

Table 3: Qualitative phytochemical analysis of methanolic extract of stem of *C. quadrangularis.*

No	Phytoconstituents	Tests	Inference
1	Alkaloids	Mayer's test	-
		Dragendroff's test	-
		Hager's test	+
		Wagner's test	-
2	Carbohydrate	Molisches test	-
3	Reducing sugar	Fehling's test	-
		Benedict's test	-
4	saponin	Foam test	++
		Froth test	++
5	phytosterols	Salkowski's test	++
		Libermann buchard's test	++
6	Phenolic compounds	Ferric chloride test	+
		Lead acetate test	+
7	Tannins	Ferric chloride	+
8	flavanoids	Lead acetate test	+
		Alkaline reagent test	+
9	Cardiac glycosides	Keller-killani test	+
10	Proteins and aminoacids	Millons test	-
		Biuret test	+
		Ninhydrin test	+
11	Terpenoids	Salkowski's test	+
12	Fixed oils and fats	Spot test	+
		Saponification test	+
13	gums and mucilages	ruthenium red solution	-

Note :++ : Higher + :Lower - :Absent

4. Conclusions and the Outlook

The methanolic stem extract was analysed for various phytochemical constituents using standard procedures and revealed the presence of alkaloid, saponins, phytosterols, phenolic compounds, flavanoids, terpenoids, protein, amino acids, fixed oils, fats, tannins and cardiac glycosides. Further studies are in progress to elucidate the wide pharmacological activity of this plant and also the reported work could be further useful to the structural elucidation and further quantification of bioactive compounds.

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