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Evaluation of phytochemical constituents and proximate contents of the ethanolic leaf extract of *Tetrastigmaleucostaphylum* (Dennst.) Alstone (Vitaceae) found in Western Ghats of Kerala, India

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Abstract

The presentstudy aims at determining the phytochemicaland proximate contents of the leaves of Tetrastigmaleucostaphylum (Dennst) Alstone (family: Vitaceae). The leaf of the plant is being commonly used by tribals of Wayanad for its various medicinal properties. The ethanolic leaf extract was analysed for various phytochemical constituents using standard procedures. The analysis of ash, crude fibre, crude protein, carbohydrate, crude fat, dry matter and moisture content of the leaves were carried out using standard proximate analysis techniques. The leaves were found to contain phytochemicals such as alkaloids,phytosteroids, saponin, diterpenes, cardiac glycosides, carbohydrate, fixed oils and fats.The result of the proximate contents showed that the dry matter has maxima of 93.15%, the moisture content 6.85%, carbohydrate 58.97% while crude fibre has a percentage of 12.00%, ash 13.78%, crude protein 17.50% and crude fat 2.90%.

Keywords: *Tetrastigmaleucostaphylum,*ethanolic extract, phytochemistry, proximateanalysis.

Introduction

Medicinal plants are a source of great economic value in the Indian subcontinent. Nature has bestowed a very rich botanical wealth with diverse types of plants in different parts of the India. The significant role of plants in maintaining human health and improving the quality of human life is known for centuries.According to the reports of World Health Organization (2005), 80 percent of the earth's inhabitants rely on traditional medicine for their primary health care needs and most of this therapy involves the use of plant extracts or their active components.Moreover, these plants and their components are alleged as “natural” and “safe” by their users. Many modern drug molecules are of plant origin. Even now one of the major sources of novel drug molecule in the development of newer drugs can be attained only with plant and for that the elucidation of phytochemical composition is highly essential. The active compounds present in the plant are responsible for their beneficial medicinal effects. This could be due to effects of a single compound or combination of one or more. In plants, the compounds with pharmacological property are mostly the secondary metabolites such as alkaloids, steroids, tannins, and phenol compounds, which are synthesized and deposited in specific parts or in all parts of the plant. They may exert their action by either mimicking or antagonizing the endogenous metabolites, ligands, hormones, signal transduction molecules or neurotransmitters and thus have beneficial medicinal effects on humans / animals due to similarities in their potential target sites[1].

Tetrastigmaleucostaphylum (Dennst.) Alstone is a woody climber. The genuscovers 100 species and belongs to grape family; Vitaceae[2]. *Tetrastigma*species arereportedly found only in subtropical and tropical region of Asia, Malaysia and Australia, where they grow in primary undistributed rainforest[3]. Species of these plants are the sole hosts of the parasitic plants in the family Raffesiaceae,which produces the largest flower in the world[4]. Some species of the genus are considered is to have important tropical medicinal properties as claimed by the locals in Indonesia and Malaysia and Vietnam, where the leaf poultice/extracts are used either internally or externally to treat headache and fever[5]. Other species *viz*., *T. hemsleyanum*is alleged to possessantipyretic, analgesic, anti-inflammatory, detoxifyingproperties besides improving blood circulation;*T. hypoglaucum*isused for the therapy of fracture, traumatic injury and swelling pain[6]. In addition, certain other species of *Tetrastigma*are also stated to be used for treatingfebrile convulsion, pneumonia, sore throat, asthma, hepatitis, rheumatism, menstrual disorders, scrofula, immune system disorders and cancer [7].

The common name of *Tetrastigmaleucostaphylum*is Indian Chestnut Vine. Traditionally, *T.leucostaphylum* (Dennist.) Alstone (figure 1) also finds use for some medicinal applications among the tribal folklore in Wayanad district. However, there is no reporton phytochemicals and proximate contents of this species.Information regarding the active components responsible for its medical propertiesis also not available. The valuable information concerning the

pharmacological properties can be obtained following extraction, isolation and purification of components from its leaves and other parts. Hence, the proposed study would help to get more insight into the active components and its medicinal applications.

Figure-1
Photograph of *Tetrastigmaleucostaphylum* (Dennist.) Alstone. in wild

Material and Methods

Plant collection and identification: The taxonomically identified *T.leucostaphylum* (Dennist.) Alstone (Vitaceae) plant leaves used in this study were collected in May, 2012 from “Chendakuni” near Meenangadi,Wayanad, Kerala, India. The plant part (leaves) was identified and authenticated by a botanist in the Department of Botany, Calicut University. A herbarium for morphological studies was prepared and avoucher specimen (CALI- 6771) deposited at Calicut University Herbarium, Kozhikode, Kerala.

Preparation of ethanolic extract: The collected plant leaves were cleaned and washed with running water and dried at room temperature for two weeks. The dried leaves were powdered in plant sample grinder at controlled temperature. The powderedleaves were used for extraction using ethanol in a Soxhlet extraction apparatus attached with a rotary vacuum evaporator (M/s Buchi, Switzerland). 100 g of powdered plant leaves was kept in filter paper thimbles (Whatman) and placed in an extraction chamber of Soxhlet apparatus and extracted using ethanol for eight cycles. Solvents were removed using rotary vacuum evaporator at 175 mbar at a temperature in the range of 40^0C to 60^0C. The weight of the dried extract was recorded and the extractive yield was calculated as,

$$\text{Extractive yield} = \frac{\text{Weight of the extract} \times 100}{\text{Weight of the sample taken}}$$

Phytochemical screening: Phytochemical screening tests for secondary metabolites were carried out on the ethanolic leafextractusingstandard procedure to identify the constituents by characteristic colour changeas described below[8-11].

Detection of alkaloids: Extractwas dissolved individually in dilute hydrochloric acid and filtered. The filtrate was further tested with following reagents for the presence of alkaloids.

Dragendroff’s Test: Filtrate was treated with potassium bismuth iodide solution (Dragendroff’s reagent). Formation of orange red precipitate indicated the presence of alkaloids.

Hager’s Test: Filtrate was treated with saturated aqueous solution of picric acid (Hager’s reagent). Presence of alkaloids wereconfirmed by the formation of yellow coloured precipitate.

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.

Wagner’s Test: Filtratewas treated with iodine in potassium iodide solution (Wagner’s reagent). Formation of reddish brown precipitate indicated the presence of alkaloids.

Detection of carbohydrates: Ethanolicextractwas dissolved in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

Molisch’s Test: Filtrate was treated with 2 drops of alcoholic α-naphthol solution in a test tube, shaken and conc. sulphuric acid added from the side of the test tube. Development of aviolet ring at the junction of two liquid confirmed the presence of carbohydrates.

Detection of reducing sugars: Benedict’s test: Filtrate was treated with Benedict’s reagent and boiled in a thermostatic water bath for 5 minutes.Formation of an orange red precipitate indicated the presence of reducing sugars.

Fehling’s Test: Filtrate was acidified with dil. hydrochloric acid, neutralized with alkali and heated with Fehling’s A & B solutions. Formation of red precipitate indicated the presence of reducing sugars.

Detection of saponins: Froth Test: The ethanolic leaf **e**xtract was diluted with distilled water to 20ml and shaken in a graduated test tube for 15 minutes. Formation of 1 cm layer of foam indicated the presence of saponins.

Foam Test: Small quantity of the extract was shaken with 2 ml of water. Persistence of foamproduced for ten minutes indicated the presence of saponins.

Detection of phytosterols: Small quantity of extract dissolved in 5 ml of chloroform was subjected to Salkowski's and LibermannBurchard'stests for detection of phytosteroids.

Salkowski's Test: On adding afew drops of conc. sulphuric acid and allowing the solution to stand, formation ofbrown ring indicated the presence of phytosterols.

LibermannBurchard's test: The chloroform extracted solution was treated with few drops of acetic anhydride, boiled and cooled. On adding conc. sulphuric acid,formation of a bluish green colour solution confirmed the presence of phytosterols.

Detection of phenolic compounds: Ferric Chloride Test: The ethanolic leaf **e**xtract was treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicated the presence of phenols.

Lead Acetate Test: The ethanolic leaf extractwas treated with 3 ml of 10% lead acetate solution. A bulky white precipitate indicated the presence of phenolic compounds.

Detection of tannins: About 0.5 g of the dried powdered plant material wasboiled in 20 ml of water in a test tube and then filtered. On adding a few dropsof 0.1% ferric chloride,development of a brownish greenor a blue-black colouration indicated the presence of tannins.

Detection of flavanoids: Alkaline Reagent Test: The ethanolic leaf **e**xtractwas treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on further addition of dilute acid, indicated the presence of flavonoids.

Lead acetate Test: The ethanolic leaf **e**xtract was treated with few drops of lead acetate solution. Formation of yellow precipitate indicated the presence of flavanoids.

Ferric chloride Test: Addition of a few drops of ferric chloride solution to the ethanolic leaf extract solution resulted in the development ofintense green colour.

Detection of proteins and amino acids: The ethanolicextract solution (100 mg in 10 ml of distilled water)was filtered through Whatman No.1 filter paper.The filtrate is tested for the presence of protein and amino acids.

Millon's Test: The test solutionistreated with few drops of Millon's reagents.A white precipitate is formed which when warmed changes to a brick red or disappears.

Biuret Test: The test solution when treated with few drops of 2% of copper sulphate solution and added 1ml of ethanol followed by excess of potassium hydroxide pellets led to the formation of pinkcolour in the ethanoliclayer.

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

Detection of terpenoids: Salkowski test: The ethanolic extract wasmixed with 2 ml of chloroform, and concentrated sulphuric acid (3 ml) wascarefully added to form a layer. A reddish brown colouration of theinterface indicated the presence ofterpenoids.

Detection of cardiac glycosides: Keller-Killani test: The ethanolicextract was treated with 2 ml of glacial acetic acid containing onedrop of ferric chloride solution. On addition of 1 ml ofconcentrated sulphuric acid, appearance of brown ring at the interface indicateda deoxysugar characteristic of cardenolides. Appearance of a violet ring below the brown ring and a greenish ring in the acetic acid layer confirmed the results.

Test for fixed oils and fats: Spot Test: Small quantity of the extract is placed between two filter papers. Oil stain produced with any extract showed the presence of fixed oils and fats in the extracts.

Saponification test: A few drops of 0.5N alcoholic potassium hydroxide added to the extract with few drops of phenolphthalein solution and heated on a water bath for 1-2 hours. Formation of soap indicated the presence of fixed oils and fats in the extracts.

Test for gums and mucilages: Smallquantity of the ethanolicextract is diluted with water and to it ruthenium red solutionwas added. A pink colour production showed the presence of gums and mucilages.

Determination of proximate contents: The analysis of ash, crude fibre, crude protein, carbohydrate, crude fat, dry matter and moisture content of the leaves were carried out using standard proximate analysis techniques[12].

Determination of dry matter: The dry matter was determined using the weight difference method and estimated by deducing percent moisture from hundred as described by James.

Dry matter (%) = 100 - % of moisture

Determination of total ash contents: Ash, representing the inorganic matter content of the sample isdetermined by the method of AOAC. Approximately one gram of thedried sample in a cruciblewas charred over a low flame and kept in a muffle furnace set at 550-600°c for 2-3 hours. It was cooled in adesiccator and weighed to ensure completion of ashing. It was heated again in the furnace for half an hour, cooled and weighed. This was repeated consequently till the weight become constant (ash became white or greyish).Total ash content was calculated by the following formula.

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

Determination of crude fat: One gram of crushed dried sample was taken in the paper thimble kept in a pre-weighed flask of fat extractor. 80 ml of petroleum ether was poured on the flask and refluxed for 8hours. The flask was cooled in a desiccator and the weight of crude fat extracted was taken. The percent crude fat was determined by using formula.

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

Determination of crude fibre: One gram of the defattened plant material was taken in aspoutless beaker and boiled in 200 mL of 1.25% sulphuric acid for 30 minutes. The content was then filtered and washed with hot distilled water to neutralize and transferred again to the beaker and boiled in 200 mL of 1.25% sodium hydroxide for 30minutes. It was again filtered and washed with hot distilled water for neutralization. The spoutless beaker was dried in ahot air oven at $100 \pm 5^{o}C$ overnight (10-12 hr), then cooled in a desiccator and weighed to a constant weight. Then, the spoutless beakers with its content wereput in a muffle furnace at $550\text{-}600^{0}C$ for 2-3 hour for complete burning of organic matter and cooled in a desiccator and weight to a constant weight. The percent fibre was determined from the formula.

$$\text{Crude fibre (\%)} = \frac{W1 - W2}{\text{Weight of sample}} \times 100$$

Where; W1- Thespoutless beaker having crude fibre was cooled and weighed, W2- The content of the spoutlessbeaker was ignited over a low flame until charred and then kept in a muffle furnace and weighed.

Determination of crude protein: Total nitrogen (N) content is determined with the help of Kjeldahl method described by Pearson (1976). The protein determination is divided into three steps. The percent of protein was calculated by the formula.

$$\text{Protein (\%)} = \frac{V \times 1.4 \times 6.25 \times 0.1N\ \text{Hcl} \times \text{Vol (used)}}{W \times A \times 1000} \times 100$$

Where; V -Titter 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.

Determination of Carbohydrate: Determination of available carbohydrate in the sample was calculated by using this formula.

Carbohydrate (%) = 100 - (moisture + crude fat + ash+crude protein) %.

Result and Discussion

The phytochemical characteristics and proximate analysis of ethanolic extract of *T. leucostaphylum* investigated are summarised in table 1 and figure 2 respectively. The ethanolic extract indicated the presence of alkaloids, phytosteroids, saponins, terpenes, cardiac glycosides, carbohydrate, reducing sugars, fixed oils and fats. The leaf extract however, did not reveal the presence of tannins, flavanoids, phenolic compounds, protein, amino acids, gums and mucilages. Nery and co-workers (2008) had detected the presence of alkaloids and phenolic compounds in the root and stem bark extracts of *T. leucostaphylum* (Dennist.) Alsone ex Mabb. actinghost toRaffesiaceae. Yang *et al.*, Liu and Yang, and Liu *et al*. had observed the chemical constituents of *T. hemsleyanum.* In another study performed by Liu *et al*., ten chemical compounds were isolated from *T. hypoglaucum.*A preliminary survey on phytochemistry of five *Tetrastigma*species from Sabah, East Malaysia, showed that *T. dubium* Planch., *T. hookeri* Planch. and*T. pedunculare*Planch. gave a positive reaction of saponins, while *T. diepenhorstii* (Miq.) Latiff and *T. glabratum*Planch. gave a negative reaction. All of them had a negative reaction of alkaloids and steroids[13]. In addition, the petroleum ether extract of *T. thomsonianum planch* showed the presence of phenolic compounds and flavanoids[14] and it is commonly used as vegetable and known to have medicinal properties[15].

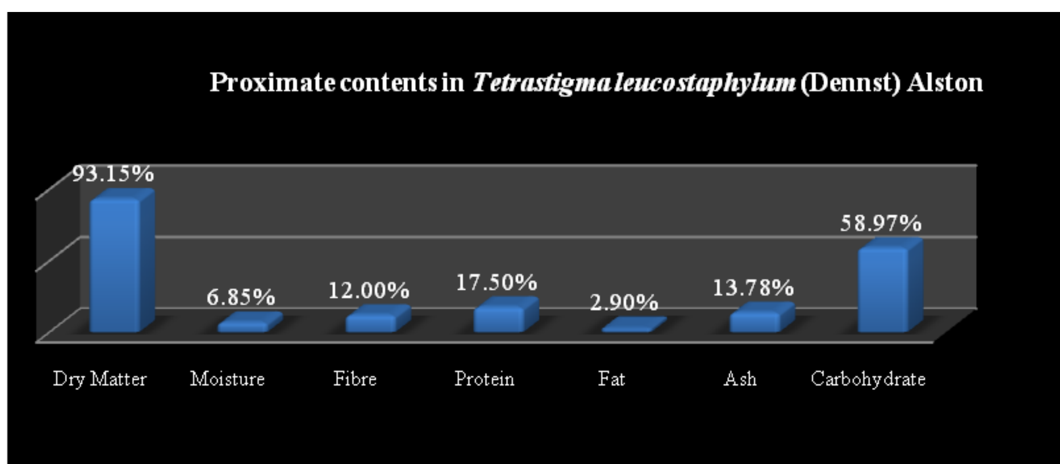

Figure-2
The proximate contents (%) of theethanolic leaf extract of *Tetrastigmaleucostaphylum* (Dennist.) Alstone

Table 1
Phytochemical constituents of the ethanolicleaf extract of*Tetrastigmaleucostaphylum*(Dennst.) Alstone.

SL.No.	Phytoconstituents	*Tests*	Inference
1	Alkaloids	Mayer's test	++
		Dragendroff's test	+
		Hager's test	++
		Wagner's test	++
2	Carbohydrates	Molisch's	+
3	Reducing sugars	Fehling's test	+
		Benedict's test	+
4	Saponins	Foam test	++
		Forth test	+
5	Phytosteroids	Salkowski's test	++
		LibermannBurchard's test	+
6	Phenols	Ferric chloride test	-
		Lead acetate test	-
7	Tannins	Ferric chloride	-
8	Flavanoids	Lead acetate test	-
		Alkaline reagent test	-
9	Cardiac glycosides	Killer-Kallani test	++
10	Protein &amino acids	Millons test	-
		Biuret's test	-
		Ninhydrin's test	-
11	Terpenoids	Salkowski's test	+
12	Fixed oils and fats	Sport test	+
		Saponification test	+
13	Gum and mucilage	Ruthenium red solution	-

Note: (++) Higher, (+) Lower and (-) Absent

The analysis of dry matter, moisture, crude fibre, crude protein, crude fat, ash and carbohydrate contents of the leaves of *T. leucostaphylum* were carried out using standard proximate analysis techniques. The results revealed that dry matter content was 93.15%, the carbohydrate content 58.97% and crude fibre was 12.00%. The ash, crude fat, and moisture percentages were 13.78%, 2.9% and 6.85% respectively. There are no further reports to support the findings.

In contrast to the phytochemical constituents of the other species of the genus *Tetrastigma* reported, high alkaloid content was detected in the leaf extract of *T.leucostaphylum.* Since alkaloids are important phytoconstituent found in plants as evidenced by various pharmacological applications as anaesthetics, CNS stimulants[16] etc., isolation and characterisation of the alkaloid content could unveil new lead in the drug discovery process.

Conclusion

The ethanolicleaf extract of *T. leucostaphylum* depicted the presence of phytochemicals such as alkaloids, phytosteroids, saponin, diterpenes, cardiac glycosides, carbohydrate, reducing sugars, fixed oils and fats. The leaf extract also showed the presence of high content of alkaloid. The moisture, ash, crude fibre, crude protein, crude fat, carbohydrate and dry matter is showed that 6.85%, 12.00%,17.5%, 2.90%, 58.95% and 93.15% respectively. Further studies are in progress to find the pharmacological properties of each active principle.

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