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

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Abstract

The present study aims at determining the phytochemical and proximate contents of the leaves of *Tetrastigma leucostaphylum* (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: *Tetrastigma leucostaphylum*, ethanolic extract, phytochemistry, proximate analysis.

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.

*Tetrastigma leucostaphylum* (Dennst.) Alstone is a woody climber. The genus covers 100 species and belongs to grape family; Vitaceae². *Tetrastigma* species are reportedly found only in subtropical and tropical region of Asia, Malaysia and Australia, where they grow in primary undistributed rainforest³. Species of these plants are the sole hosts of the parasitic plants in the family Rafflesiaceae, which produces the largest flower in the world⁴. Some species of the genus are considered 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 externally or externally to treat headache and fever⁵. Other species *viz.*, *T. hemsleyanum* alleged to possess anti-inflammatory, analgesic, anti-inflammatory, detoxifying properties besides improving blood circulation; *T. hypoglauca* is used for the therapy of fracture, traumatic injury and swelling pain⁶. In addition, certain other species of *Tetragamone* are also stated to be used for treating febrile convulsion, pneumonia, sore throat, asthma, hepatitis, rheumatism, menstrual disorders, scrofula, immune system disorders and cancer⁷.

The common name of *Tetrastigma leucostaphylum* is Indian Chestnut Vine. Traditionally, *T. leucostaphylum* (Dennst.) Alstone (figure 1) also finds use for some medicinal applications among the tribal folklore in Wayanad district. However, there is no report on phytochemicals and proximate contents of this species. Information regarding the active components responsible for its medicinal properties 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 Tetrastigma leucostaphyllum (Dennist.) Alstone. in wild*

**Material and Methods**

**Plant collection and identification:** The taxonomically identified *T. leucostaphyllum* (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 auvoucher 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 powdered leaves 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°C to 60°C. 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 leaf extract using standard procedure to identify the constituents by characteristic colour change as described below.

**Detection of alkaloids:** Extract was 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 were confirmed 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:** Filtrate was treated with iodine in potassium iodide solution (Wagner’s reagent). Formation of reddish brown precipitate indicated the presence of alkaloids.

**Detection of carbohydrates:** Ethanol extract was 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 an violet 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 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.
- **Foam Test:** Small quantity of the extract was shaken with 2 ml of water. Persistence of foam produced 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’tests 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 extract 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 was boiled in 20 ml of water in a test tube and then filtered. On adding a few drops of 0.1% ferric chloride, development of a brownish green or a blue-black colouration indicated the presence of tannins.

Detection of flavanoids: Alkaline Reagent Test: The ethanolic leaf extract was 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 extract was treated with few drops of lead acetate solution. Formation of yellow precipitate indicated the presence of flavonoids.

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

Detection of proteins and amino acids: The ethanolic extract 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 solutionis treated 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 1 ml of ethanol followed by excess of potassium hydroxide pellets led to the formation of pink colour in the ethanolic layer.

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

Detection of cardiac glycosides: Keller-Killani test: The ethanolic extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. On addition of 1 ml of concentrated sulphuric acid, appearance of brown ring at the interface indicated deoxy sugar 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: Small quantity of the ethanolic extract is diluted with water and to it ruthenium red solution was 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.

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 is determined by the method of AOAC. Approximately one gram of the dried sample in a crucible was charred over a low flame and kept in a muffle furnace set at 550-600°C for 2-3 hours. It was cooled in desiccator 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:

\[
\text{Ash} (%) = \frac{\text{Weight of ashed sample} \times 100}{\text{Weight of sample taken}}
\]
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 8 hours. 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.

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

Determination of crude fibre: One gram of the defatted plant material was taken in a spoutless 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 30 minutes. It was again filtered and washed with hot distilled water for neutralization. The spoutless beaker was dried in a hot air oven at 100±5°C overnight (10-12 hr), then cooled in a desiccator and weighed to a constant weight. Then, the spoutless beakers with its content were put in a muffle furnace at 550-600°C for 2-3 hours 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.

\[
\text{Crude fibre (\%)} = \frac{W_1 - W_2}{\text{Weight of sample}} \times 100
\]

Where; \(W_1\) - The spoutless beaker having crude fibre was cooled and weighed, \(W_2\) - The content of the spoutless beaker 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.

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

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.

\[
\text{Carbohydrate (\%)} = 100 - (\text{moisture + crude fat + ash+crude protein}) \%
\]

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.) Alstone ex Mabb, acting host to *Rafflesiaceae*. 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. leucostaphylum*. 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. In addition, the petroleum ether extract of *T. thomsonianum* planch showed the presence of phenolic compounds and flavanoids and it is commonly used as vegetable and known to have medicinal properties.
Table 1

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

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 etc., isolation and characterisation of the alkaloid content could unveil new lead in the drug discovery process.

**Conclusion**

The ethanolic leaf 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.

**Acknowledgement**

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