5. Fingerprint Profile of an important therapeutic plant of Astavarga *Crepidium acuminatum* (D. Don) Szlach by HPTLC

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Abstract
Nature is crucial source of drugs. Plants have played important role in identifying important drugs and are basis of various modern pharmaceuticals. Therefore ethnomedicinal plants must be exploited to identify lead compounds. *C. acuminatum* is an important plant of Astavarga (combination of 8 drugs, most commonly used in Ayurveda) *C. acuminatum* is used in breathing disorders, burning sensation, cough, decrease in bone tissue, bleeding disorders, blood disorders, tuberculosis, insect bites and rheumatism. It is refrigerant, febrifuge and aphrodisiac. It is utilized as tonic and in general debility. HPTLC is a valuable analytical tool for the investigation of herbal products and drugs. Hence, HPTLC Fingerprint profile has been developed for this plant.

Objective: The present study was aimed to develop the fingerprint profile by HPTLC of *Crepidium acuminatum* (D. Don) Szlach.

Materials and Methods: HPTLC System (CAMAG, Switzerland), equipped with linomat 5 applicator, development chamber, scanner, derivatizer, vision Cats software, was used. The plate was scanned at 580 nm using Tungsten lamp and images were captured at visible light, UV 254 nm and UV 366 nm.

Results: HPTLC method for separation of phytoconstituents using different solvent system has been developed for *C. acuminatum*. The study revealed the presence of 2 saponins, 3 bitter principles, 2 steroids, 2 Sterols, 2 essential oils, 1 anthraquinones, 2 coumarin and 5 flavonoids in methanol extract of pseudobulbs of this plant.

Conclusion: The HPTLC fingerprint profile developed for methanol extract of *C. acuminatum* can be used for routine quality control of herbal formulations comprising of this plant and serve as a base for qualitative, quantitative analysis and standardization of the drug ‘Jeevak’. It will also help in identification and quantification of active/ marker compounds. By isolating and identifying marker compounds, new drugs can be formulated to treat various diseases.

Keywords: HPTLC, Fingerprint profile, *C. acuminatum* (Jeevak), *Malaxis acuminata* Astavarga, Phytochemical analysis

Introduction
Traditional plant medicine is becoming an area of ever-increasing importance in the health care systems. Since times immemorial, plants form the basis of various traditional therapeutic systems like, Ayurveda, Unanai, Sidha. Uses of plant based remedies in healthcare preparations have been reported in Vedas and the Bible. Plants produce a diverse group of bioactive molecules, making them a rich source of different types of medicines (1). These days medicinal and neutraceutical herbs are receiving immense scientific attention for their holistic effects (2). Thus, natural products with pharmacological or
biological activities are playing a very important role in medicine (3). World Health Organization (WHO) has confirmed that herbal medicines are serving the health needs of about 80 percent of the world’s population especially in rural areas of developing countries. Attention has also been paid due to the side effects of most modern drugs. It has been estimated that in the mid-1990s over 200 companies and research organizations worldwide were screening plant and animal compounds for medicinal properties (4, 5). Important drugs like vinblastine, vincristine, topotecan, taxol, teniposide, etopside, irinotecan etc. have come from plant sources. Curiosity is escalating in the overall fitness & wellness of man due to nutraceutical plants.

*Crepidium acuminatum* (D.Don) Szlach (Syn. *Malaxis acuminata*) is having immense ethnomedicinal potential. The dried pseudobulbs known as ‘jeevak’ are important ingredients of various formulations and a polyherbal immune-booster nutraceutical ‘Chyavanprash’. This drug has been stated in various ayurvedic formulations like Astavarga churna, chyavanprash Rasayan, Ghrita, Taila, Gutika, Agada etc. (6). *C. acuminatum* is used in breathing disorders, burning sensation, Cough, decrease in bone tissue, bleeding disorders, blood disorders, tuberculosis, insect bites, rheumatism. It is reported to be refrigerant therefore used to reduce fever (Febrifuge). It has been described as aphrodisiac and used in emaciation, seminal weakness. It is utilized as tonic and in general debility (7, 8, 9) Although its antimicrobial (10), antioxidant (11) essential oil analysis (12), and anti-inflammatory activities (13) have been reported but work has not been described on fingerprint profile of this plant by HPTLC.

High Performance Thin Layer Chromatography (HPTLC) is a sophisticated analytical technique pedestal on the full potential of thin layer chromatography. Automation, scanning, full optimization, selective detection principle, minimum sample preparation, hyphenation enable it to be a powerful tool in modern research for qualitative and quantitative analysis of complex mixtures of bio molecules (14). It has gained popularity to become a leading type of analysis in fingerprinting of herbal drugs. Although thin layer chromatography (TLC) is commonly used for the analysis of herbal drugs since long time back. Various pharmacopoeias such as American Herbal Pharmacopoeia (AHP), Chinese Drug Monographs and Analysis, Ayurvedic pharmacopoeia of India (API) Pharmacopoeia of People’s Republic of China, etc. still use TLC to provide first characteristic fingerprints of herbal drugs. Fingerprint analysis approach using chromatography is becoming the most powerful tool for identification, authentication and quality control of herbal products. For quality control, the concept of phyto-equivalence is utilized. Chromatographic fingerprint can demonstrate ‘sameness’ and ‘differences’ between various formulations and the authentication and identification of herbal medicines can be accurately carried out even if the number and concentration of chemically characteristic components are not very similar in different samples of herbal formulations (16). As per World Health Organization (WHO), the quality, quantity, safety and efficacy data on traditional medicine are not sufficient and there is still a lack of adequate/accepted research methodology for evaluating traditional medicine till date. In this situation, HPTLC is playing pivot role for the fingerprinting of medicinal plants. It is a realistic alternative to meet the need for effectual and powerful assessment of herbal products. HPTLC is mainly used for expressing various patterns which are preserved as ‘databases’ known as ‘fingerprints’ for future studies. In such a situation, there is dire need that chromatographic fingerprint, for a herbal product should be constructed. Hence, this technique was adopted for deriving the fingerprint patterns of the crude drug of *Crepidium acuminatum*.

**Materials and Methods**

Pseudobulbs of the plant were screened for profiling. Pseudobulbs were shade dried in air at room temperature, powdered and stored in air.
tight container. It was authenticated and identified as *Crepidium acuminatum* (D. Don) Szlach from National Institute of Science Communication and Information Resources (NISCAIR), New Delhi and deposited in herbarium of Panjab University, Chandigarh with PAN 21262.

For analysis 500mg of powder was dissolved in 10 ml of methanol with occasional shaking for duration of 2 hrs., then filtration was done by using membrane filter. Sample of 1, 2, 4, 6 μl aliquot were loaded as 8 mm band length on a 5 X10 silica gel 60 F 254 TLC plate using LINOMAT 5 auto sampler instrument (CAMAG, Muttenz, Switzerland). The samples-loaded plates were kept in TLC twin trough developing chamber after saturation, (saturation time 5 min) with solvent vapour using respective mobile phases (as shown in table 1) and the plates were developed in the respective mobile phases up to 70 mm. The developed plates were dried by hot air to evaporate solvents from the plate. The plates were kept in a photo-documentation chamber (CAMAG) and the images were captured. Software Visioncats-serv, version 2.4.17207.2 was used for data analysis.

**Results**

The present study, which was performed to develop fingerprint of the drug by using HPTLC technique showed valuable results. 2 saponins, 3

<table>
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<th>S. No.</th>
<th>Class of Compounds studied</th>
<th>Mobile Phase/composition</th>
<th>Derivatization Reagent</th>
<th>Result &amp; Interpretation</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
<td>Saponins</td>
<td>Chloroform: acetic acid: methanol: water/6.4:3.2:1.2:0.8</td>
<td>Anisaldehyde sulphuric acid reagent</td>
<td>Band at Rf 0.80 and 0.50, violet color shows presence of saponins.</td>
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<tr>
<td>2.</td>
<td>Bitter Principle</td>
<td>ETHYL acetate: methanol; water/7.7:1:5:0.8</td>
<td>No</td>
<td>Band at Rf 0.37, 0.60 and 0.90, bluish violet color indicates presence of bitter principles.</td>
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<td>3.</td>
<td>Essential oils</td>
<td>Toluene: ethyl acetate/9.3:0.7</td>
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<td>4.</td>
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<td>Natural product Reagent</td>
<td>Fluorescence at Rf 0.10, 0.25, 0.75, 0.80, 0.97 indicates presence of flavonoids.</td>
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<tr>
<td>8.</td>
<td>Steroids</td>
<td>n-butanol: methanol: water/3:1:1</td>
<td>Anisaldehyde sulphuric acid reagent</td>
<td>Band at Rf 0.60 and 0.67 red-violet color indicates presence of steroids.</td>
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bitter principles, 2 steroids, 2 sterols, 2 essential oils, 1 anthraquinones, 2 coumarin and 5 flavonoids were observed in methanol extract.

Methanol extract was subjected to different composition of the mobile phase (Table 1) to separate different secondary metabolites. After derivatization with appropriate reagents, the colour development was noted. Based on colour development and fluorescence (15), the secondary metabolite were differentiated and RF values were calculated and presented in figure. Best solvent system for phytochemical analysis are presented in table 1.

HPTLC fingerprinting studies of methanol extract showed distinct band pattern before and after spraying with derivatizing reagent. RF values under different wavelengths before and after derivatization are taken and presented in figure 1-8 and Table 1. HPTLC, now a days is applied not only to obtain “Fingerprint” patterns of herbal formulations, quantification of active ingredients but also for the detection of adulterant.

Discussion
The preliminary phytochemical screening of crude drug indicated the presence of saponins, essential oils, anthraquinones, Sterols, coumarins, flavonoids, steroids, tannins and glycosides by HPTLC.

Saponins are a large family of phytochemicals which are structurally related compounds. ‘Sapo’ is a Latin word for soap. They possess soap like properties and form lather in aqueous solution. They possess surfactant properties and are used as natural detergent. Chemically they are glycosides of steroids (C27) and triterpenes (C30). Saponin = glycone + aglycone. Glycone is sugar moiety, which is polar in nature and consists of hexoses/pentoses/uronic acid, whereas aglycone part, is known as sapogenin, steroidal/triterpene. They have received industrial, commercial, pharmaceutical attention. They are used as food additives, as ingredients in photographic emulsions, in fire extinguishers etc. (17) Biological effects of saponins are in the membrane-permeabilising, immunostimulant, hypcholesterolaemic and anticarcinogenic properties. They have also been found to affect significantly reproduction in animals. These structurally diverse compounds have also been observed to kill protozoans and molluscs, to have an effect on cold blooded animals, also to have the analgesic, antinoceceptive, antioxidant activity, to impair the digestion of protein, to cause hypoglycemia and to act as antifungal and antiviral agents (18). Investigations are going on towards the development of new natural medicines and prove the efficacy of traditional herbal medicine. The plant under investigation showed two types of saponins at Rf 0.80 and 0.50 which can be isolated and characterized for further studies.

The term ‘essential oil’ derives its name from the drug *Quinta essentia*, named by Paracelsus von Hohenheim of Switzerland in sixteen century. Essential oils are ‘essences’ which are responsible for different scents that plants emit. They are odours, flammable, volatile products which have tendency to evaporate on exposure to air even at ambient conditions and therefore also referred to as volatile oils or ethereal oils. Essential oils are indispensable in food, cosmetic and human health field. They are extensively used in perfumery and aromatherapy (therapeutic technique including massage, inhalations, or baths using these volatile oils). They serve as chemical signals allowing the plant to control or regulate its environment (ecological role), attraction of pollinating insects, repellent to predators, inhibition of seed germination, and communication between plants. They have antibacterial, antioxidant, anti-inflammatory, cancer chemoprotective activity, antifungal or insecticide and deterrent activities etc. (19, 20).

Anthraquinones, another class of phytochemicals, are aromatic compounds having anthracene ring with 2 keto groups (9, 10-dioxoanthracene). These are derivatives of phenolic and glycosidic compounds but in living plants they are generally found as glycosides. They are derived from anthracene and giving variable groups based on the degree of oxidation.
and position of double bonds in the polycyclic and side chain systems of sterols can be different. Generally, the sterols can be categorized into three subclasses: (I) 4, 4 desmethylsterols (II) 4a-methylsterols and (III) 4, 4-dimethylsterols. Phytosterols are important products for health and nutrition industries. They have hypocholesterolemic activities, and are used as cholesterol-lowering agents contributing towards cardiac health benefits. They are useful emulsifiers for cosmetic manufacturers and used as precursors for the production of hormones. They are known to inhibit oxidative deterioration of oils, therefore serving as potential antipolymerization agents for frying oils and used as markers for the assessment of adulterated oils. Sterols are present in various botanical sources, such as fruits, seeds, and vegetables. They are particularly rich in legumes, nuts, and seeds. Sterols are known to have important health benefits and are widely used in the food and pharmaceutical industries.

Sterols are a class of phyto-compounds, derived from hydroxylated polycyclic isopentenoids which is having a 1,2-cyclopentanophenthrene structure. These compounds contain a total of 27-30 carbon atoms in which a side chain with carbon atoms is attached at the carbon 17 position. The number and position of double bonds in the polycyclic and side chain systems of sterols can be different. Generally, the sterols can be categorized into three subclasses: (I) 4, 4 desmethylsterols (II) 4a-methylsterols and (III) 4, 4-dimethylsterols. Phytosterols are important products for health and nutrition industries. They have hypocholesterolemic activities, and are used as cholesterol-lowering agents contributing towards cardiac health benefits. They are useful emulsifiers for cosmetic manufacturers and used as precursors for the production of hormones. They are known to inhibit oxidative deterioration of oils, therefore serving as potential antipolymerization agents for frying oils and used as markers for the assessment of adulterated oils. Sterols are present in various botanical sources, such as fruits, seeds, and vegetables. They are particularly rich in legumes, nuts, and seeds. Sterols are known to have important health benefits and are widely used in the food and pharmaceutical industries.

E.g., anthrones, anthranols, chrysophanol, poramide, luteolin, emodin etc. (21). They are most commonly utilized as laxatives and possess antiviral and antifungal properties. These compounds impart color to plants and have been extensively employed as natural dyes (22). One anthraquinone has been observed in methanol extract of this plant in current study.

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**Fig. 1:** represents results for saponins captured after derivatization 1 (a) under white light; arrows at band Rf 0.80 and Rf 0.50 observed under white light indicates presence of saponins (b) same plate under UV 366 nm.

**Fig. 2:** shows image representing results for bitter principles, visualized under 2 (a) white light and 2 (b) UV 366 nm arrows at bands in 2 (a) corresponding to Rf 0.37, 0.60 and 0.90 of bluish violet color indicated presence of bitter principles.

**Fig. 3:** Image obtained after derivatizing with vanillin and Sulphuric acid and observed under 3(a) white light and 3 (b) under UV 366 nm. Arrows pointing at bands corresponding to Rf 0.29 and 0.75 of violet color in 3 (a) indicated presence of different essential oils (terpenoids).

**Fig. 4:** Image acquired after derivatizing with alcoholic KOH and taken under 4(a) white light and 4(b) UV 366 nm. Arrow at band Rf 0.65 in 4 (a) showing yellow florescence is pointing towards anthraquinone.

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in the nonsaponifiable fraction of plant oils. Since these are of plant origin and not synthesized in humans therefore are poorly absorbed and are excreted faster than cholesterol. Therefore they have lipid lowering efficacy. Main phytosterols which are used in the human diet are sitosterol, stigmasterol, and campesterol. (23, 24). Two types of sterols observed in the present study at Rf 0.10 and 0.79 of reddish–violet color band confirmed the presence of sterols.

'Coumarins' word is derived from 'Coumarou', the vernacular name of the tonka bean (Dipteryx odorata), from which coumarin, was first time isolated in 1820. There are four classes of coumarin; simple coumarins; furanocoumarins; pyranocoumarins; and pyrone-substituted coumarins. The coumarins have bacteriostatic and anti-tumor activity and these compounds are being screened as novel therapeutic agents (25). Two types of coumarins are first time reported in existing study at Rf 0.05 & 0.71 showing fluorescence of pale yellow colour indicated presence of coumarins.

Flavonoids are polyphenolic compounds, ubiquitous in nature and are more than 4,000 commonly occur in vegetables, fruits and beverages like tea, coffee and fruit drinks. They occur as aglycones, glucosides and methylated derivatives. They are utilised as food and pharmaceutical supplements. The flavonoids appear to have played a major role in successful medical treatments (26). Flavonoids are linked to their potential cytotoxicity and their capacity to interact with enzymes through protein
complexation (27). They have antioxidative activity, free-radical scavenging capacity, anticancer activity and have role in coronary heart disease prevention and anti-human immunodeficiency virus functions. They are reported to be hepatoprotective, anti-inflammatory, and antiviral also (28). Five types of flavonoids are reported in present investigation carried out.

Steroids have the fundamental structure of four carbon rings called the steroid nucleus. The addition of different chemical groups at different positions on backbone leads to the formation of many different types of steroidal compounds. Plant steroids are synthesized by cyclisation of 2,3-epoxysqualene into cycloartenol, which are further metabolized to produce biologically active steroids. Plant steroids classified in different classes based on their chemical structure, pharmacological activities and source from which they have been isolated. Plant steroids possess many interesting medicinal, pharmaceutical and agrochemical activities like anti-tumor, immunosuppressive, hepatoprotective, antibacterial, plant growth hormone regulator, sex hormone, anthelmintic, cytotoxic and cardiotonic activity (29).

Chemically, it is difficult to define tannins as they include diverse number of oligomers and polymers. Tannins are a heterogeneous group of high molecular weight polyphenolic compounds which form reversible and irreversible complexes with proteins, polysaccharides (cellulose, hemicellulose, pectin etc.), alkaloids, nucleic acids and minerals etc. Structurally, tannins can be categorised into four groups: Gallotannins, ellagitannins, complex tannins, and condensed tannins. Gallotannins are all those tannins in which galloyl units or their derivatives are joined to polyol, catechin-, or triterpenoid units. Ellagitannins are those tannins in which at least two galloyl units are C–C coupled to each other, and do not contain a glycosidically linked catechin unit. Complex tannins are tannins in which a catechin unit is bound glycosidically to a gallotannin or an ellagitannin unit. Condensed tannins are all oligomeric and polymeric proanthocyanidins. Tannin-containing plant extracts are used as astringents, anti-inflammatory, as anti-septic, as diuretics, antioxidant and haemostatic. They are also used against diarrhoea and various types of tumours. Tannins are used in the dye stuff industry and also in the production of inks. Other industrial uses of tannins include textile dyes, as antioxidants in the fruit juice, beer and wine industries, and as coagulants in rubber production. Recently the tannins have attracted scientific interest for treatment of AIDS and various cancers (25, 30).

Glycoside is a generic term used for phytochemicals that are bound to a sugar. They are the compounds that yield one or more sugars upon hydrolysis. Hence the glycoside consists of two parts: the sugar and the aglycone part. The aglycon may be a terpene, flavonoid, coumarine etc. Among the sugars found in natural glycosides, D-glucose, L- rhamnose and L-fructose, L-arabinose are commonly found. The sugar part can be disaccharide also. The classification of glycosides is a difficult matter. They are usually mixed acetals. The sugar moiety of a glycoside is joined to the aglycone, according to the chemical group of the aglycone involved into the acetal union, they are O-glycoside (OH group); S-glycoside (SH group), N-glycoside (NH group), C-glycoside (C group). The systematic names are formed by replacing the 'ose' suffix of the parent sugar with "oside". Classification can be based on the sugar group for e.g., lucosides and rhamnosides, classification can be based on aglycone group, Examples are lignan glycosides, alkaloidal glycosides etc. Glycosides which show soap like properties are called saponin. Glycosides that release hydrocyanic acid on hydrolysis are known as cyanogenic glycosides. Based on functional group they can be Phenolic glycosides, Aldehyde glycosides, Anthraquinone glycosides etc. (31)

The compounds which were reported earlier in C. acuminatum are alkaloid, carbohydrates, flavonoids, resin, saponin, steroids, tannins, whereas triterpenoids have been reported negative.
qualitatively (32), whereas in same report proteins are also documented as negative. Essential oils such as Limonene, Eugenol, citronellal, 1-8-cineole, Piperitone and p-cymene were reported by TLC (33), whereas in another study beta – sitosterol, cetyl alcohol, glucose, rhamnose are reported. (34) Volatile oils are estimated as 0.54 ± 0.28(%v/w) in Tarikhet sample (32). In one recent studies (35) dietary fatty acids, alpha – hydroxy acids, phenolic acids, sterols, amino acids, sugars and glycoside are reported in this plant by Atomic absorption spectrophotometer and GC-MS. Another group of scientists (36) analysed quantitatively metal content and volatile constituents of the plant by Atomic absorption spectrophotometer and GC-MS. Alpha tocopherol and gamma tocopherol along with terpenoids are also reported (36), whereas presence of Acidic polysaccharides, Anthocyanins, Lignin, Phenolic substances, cutin, suberin, lignin and starch have been documented (37). Scientists (38) also confirmed the presence of polyphenols, flavonoids, while synthesising gold nanoparticles of the extracts of pseudobulbs. Whereas comprehensive report and fingerprint profile has been presented in this paper. These secondary metabolites are accountable for the therapeutic activity of plants (39). Hence, great potential of Crepidium acuminatum as a nutraceutical and herbal drug is confirmed in the present study.

Conclusion:

C. acuminatum is an important plant of Astavarga. It has a lot of therapeutic potential. The present study revealed the presence of saponins, bitter principles, steroids, Sterols, essential oils, anthraquinones, coumarin and flavonoids in methanol extract. The HPTLC fingerprint profile developed for methanol extract of C. acuminatum can be used for routine quality control of the drug and serve as a base for qualitative & quantitative analysis and standardization of the drug. It will also help in identification and quantification of active/marker compounds. By isolating and identifying marker compounds, new drugs can be formulated to treat various diseases.

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