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Scientific validation of secondary metabolites profile from stem part of medicinally important plant *Vincetoxicum subramanii* (AN Henry) Meve & Liede (Apocynaceae) using TLC and HPLC method

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Abstract
Secondary compounds of *V. subramanii* stem were qualitatively analyzed using TLC and HPLC. Ethanol stem extracts were prepared for qualitative determination of secondary metabolites. The resulting extracts were subjected to a variety of solvent-based systems, using conventional TLC techniques to identify secondary metabolites, followed by HPLC analysis to identify commonly available compounds such as quercetin and catechins. including alkaloids, flavonoids, phenols, tannins, saponins and glycosides. It became clear that chromatograms generated at different retention times show the presence of different plant constituents as a result of HPLC analysis. 19 peaks were identified. *V. subramanii* stem were found to contain alkaloids, flavonoids, glycosides, saponins and steroids.

Keywords: Catechin, HPLC, Quercetin, TLC, *Vincetoxicum subramanii.*

Introduction
Plants are essential in the treatment and prevention of disease and can even avoid or reduce the negative effects of standard treatments [1]. Herbs and dietary supplement additives are examples of dietary supplements. 'There are about 600,000 plant species, but only 2.5 are photochemical' [2]. They can contain biologically and pharmacologically important molecular substances. Plants have traditionally been a source of potent drugs and are still important for the evaluation of novel lead compounds [3] The discovery of bioactive substances in plants is an important part of plant research, leading to further biological and pharmacological investigations [4-6]. Advances in technology have made it possible to identify, filter and isolate these active ingredients. The use of chromatographic and spectral fingerprinting in the quality control of complex herbal medicines is of great importance [7]. The first step in identifying phytochemicals in a sample is thin layer chromatography (TLC) [8]. Herbal medicines continue to face quality control and assurance challenges as they contain numerous compounds in complex matrices without a single active ingredient responsible for their potent effects [9]. Therefore, systematic evaluation of all plant constituents is as important as measuring active ingredients. In practice, the chromatograph uses his fingerprint. Chromatographic profiles reveal multiple chemical constituents that characterize the herbal remedy under study and identify and assess chemical stability [10]. TLC fingerprinting profiles of herbal medicines provide a comprehensive qualitative method for species identification, quality assessment, and ensuring homogeneity and stability of herbal medicines and their products. High performance liquid chromatography is a more sophisticated variant of column chromatography. This technique uses a liquid mobile phase to separate sample components as they move through the column and provides statistics on compound separation. This greatly improves the separation of the components of the mixture. Available detection technology is another important advance over column chromatography. These techniques are highly accurate and highly automated. Both methods are more effective, faster, and yield more consistent and reproducible outcomes [11]. 'A literature review has revealed the invention of several spectroscopic techniques, HPTLC and RP-HPLC, for the quantitative determination of secondary metabolites' [12]. *Vincetoxicum subramanii*, also known as Subramani's Ipecac, is a member of the family Apocynaceae [13]. This species has many therapeutic uses and its common name is paal kodi [14].

Traditional physicians used the leaves of these plants as expectorants, rheumatic analgesics, and in diabetes and cancer treatments [15-17]. The whole plant has the ability to be a natural antioxidant [18]. Jaundice is treated with raw leaf juice [19]. It is well known that therapeutically important herbs contains a wide range of secondary metabolites, some of which are involved in biological effects. As a result, the purpose of this study was determined and enrich various secondary metabolites found in the parent sample using TLC and HPLC.

Materials and Methodology

Collection and Identification

The plant with reproductive parts have been collected in August 2022 from the Meghamalai hills, part of Southern Western Ghats in, Theni District of Tamil Nadu, INDIA., Taxonomic identification of plant was authenticated by Botanical Survey of India, Southern Regional Centre, Coimbatore, Tamil Nadu (BSI/SRC/5/23/2022/Tech/398) as *Vincetoxicum subramanii* (A.N Henry) Meve & Liede.

Preparation of *V. subramanii* Extract

The collected samples were cleaned and dried. The samples were then crushed and stored in containers until use. 25 g of *V. subramanii* stem powder was packed into thimbles with (Whatman) filter paper and extracted with 250 ml of ethanol using a soxhlet extractor at temperatures varying from 60 to 65 °C. for 24 hours. A rotating vacuum evaporator was used to evaporate the liquid, resulting in a viscous semi-solid substance. TLC and HPLC analyses were carried out on this semi-dried pure ethanol extract.

Qualitative analysis of secondary metabolites through TLC and HPLC analysis

TLC and HPLC experiments were performed using methods described by Harborne [20] and Wagner *et al*. [21].

TLC (Thin Layer Chromatography) profile of plant extract

Ethanol extracts of *V. subramanii* stem were carefully transferred to precoated silica gel G, TLC plates using capillary tubes to separate various phytochemical constituents (15 ×5 cm, 3 mm thick). Stained plates were immersed in solvent solutions and the correct mobile phase was identified using the technique described above [22, 23]. Spray reagents such as Dragendorff reagent, ALCL3 reagent, sodium chloride solution, folin-ciocultue reagent, KOI reagent, FeCl3 reagent, Kedde reagent and vanillin sulfate reagent were used to identify the respective compounds. The dot color was recorded and the retention factor was determined using the following formula.

$$\text{Retention time (Rf)} = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

HPLC analysis

Sample Preparation

Dissolve 100 milligrams of substance in 10 milliliters of methanol. If the material does not dissolve, filter the sample through a 0.22 µm sieve to remove impurities.

Reagents

HPLC grade water, Acetonitrile (CH3CN), HPLC, Methanol and 0.1% acetic acid Standard preparation: Standard was prepared by using methanol extract (stock concentration:100 mg/10 ml) and the most important biologically active compounds such as quercetin and catechins, were used [24, 25].

Solvent Preparation

Filter all solvents through a 0.22 µm filter before use. This prevents particles from clogging the solvent lines and columns and allowing the solvent to evaporate. If your HPLC instrument does not allow on-line degassing, check the instrument specifications to see if additional degassing is required. Sample integration and inspection. Once a stable baseline was established, 20 µl of material (manual injector) was injected and chromatographed on a waters HPLC (machine equipped) (model 299, Waters) using a photodiode array detector. graphic isolate.

Data were collected using a Waters HPLC adapter and Empower 2 software. Quercetin and Catechin was analyzed using HPLC on an RP-18e Lichrosphere® column (250.4 mm, 20 m, Shimadzu Corporation, Japan) with acetonitrile and water containing 0.1% acetic acid as mobile phases at 1.0 ml/min and washed. One flow rate was used and the wash temperature was 30.3°C. Column discharge was observed at 350 nm (PDA detection) [25]. Quercetin and catechin standards were reduced using methanol and the samples were filtered through a 0.45 µm PVDF membrane [26].

Mobile phase

Solvent A: 0.1% (v/v) acetic acid

Solvent B: Acetonitrile

Programme: A gradient elution programmed as follows: 0% B (0.1 min), 10% B-15% B (5 min), 15% B-25% B for (next 5 min), 25% B-35% B (10-15min), 35% B-50% B (15 min, 20 min).

Flow rate: 1 mL/min

Injection volume: 20 µL

Result

TLC analysis

With the help of TLC and HPLC techniques, the current study sought to identify the phytochemicals present in the *V. subramanii*

Table 1: Using TLC and the proper mobile phases, secondary metabolites in ethanol stem preparations of *V. subramanii* were examined.

Phytoconsttuents	Mobile phase	Spray reagent	Spot colour	Rf Value
Alkaloid	Chloroform: Methanol: Ethyl acetate (2:2:0.5)	Dragondorf reagent	Orange brown	0.84
Flavanoid	Ethyl acetate: Methanol: Gallic Acid (5:0.5:0.5)	$ALCL_3$ reagent/ 5% Ferric chloride solution	Yellow/Grey	0.7
Phenols	Chloroform: Methanol (27:0.3)	Folincioculteuereagemt/KOI	Blue/Green	0.6
Tannin	Methanol: water (3:15)	$Fecl_3$ reagent	Brownish green	Not detected
Saponin	Chloroform: Methanol: Water (3:0.5:0.5)	Vannilin H_2OSO_4 reagent	Violet/blue	Not detected
Glycosides	Ethyl Acetate: Methanol (4:1)	Kedde reagent	Distinct band form/ pinkish violet colour	0.75

Note: a-Before derivatization, b-After derivatization.

Fig 1: Segregation of Phytochemical constituents of ethanol stem extract of *V.*

TLC (Thin Layer Chromatography) profile

Different mobile phase compositions were tried to separate all different secondary metabolites such as alkaloids, flavonoids, phenols, tannins, saponins and glycosides by subjecting the primary ethanol extract to TLC. (See Table 1 and Figure 2). TLC plates were developed with appropriate extraction solutions before samples were measured. A tint was observed that was produced after derivatization with a suitable spray solution. Color was used to identify secondary compounds and calculate Rf values. TLC analysis reveals that the highly polar solvent ethanol extracts a broader range of pharmaceutically important secondary metabolites from the stem of the *V. subramanii* species in question, including alkaloids, flavonoids, phenols, tannins, saponins and glycosides. It became clear that two different combinations of chloroform, methanol, and ethyl-acetate in the ratio of 2:2:0.5 proved to be appropriate mobile phases for ethanolic extracts of stem for the secondary metabolite alkaloids. The study revealed that mixture made up of ethyl acetate, methanol, and gallic acid in the ratio of 5:0.5:0.5 was found to be the best mobile phase for the secondary product flavonoids. The combination of chloroform and methanol in the ratio of 27:0.3 was discovered to be a suitable mobile phase for the phenol, whilst the combination of ethyl acetate and methanol in the ratio of 4:1 was confirmed to be a suitable mobile phase for the glycosides. The Rf values obtained for the compound are listed in Table 1. The results indicate that unreasonably polar solvents such as chloroform, ethyl acetate, and methanol are the best mobile phases for separating bioactive metabolites from *V. subramanii* stem.

HPLC analysis

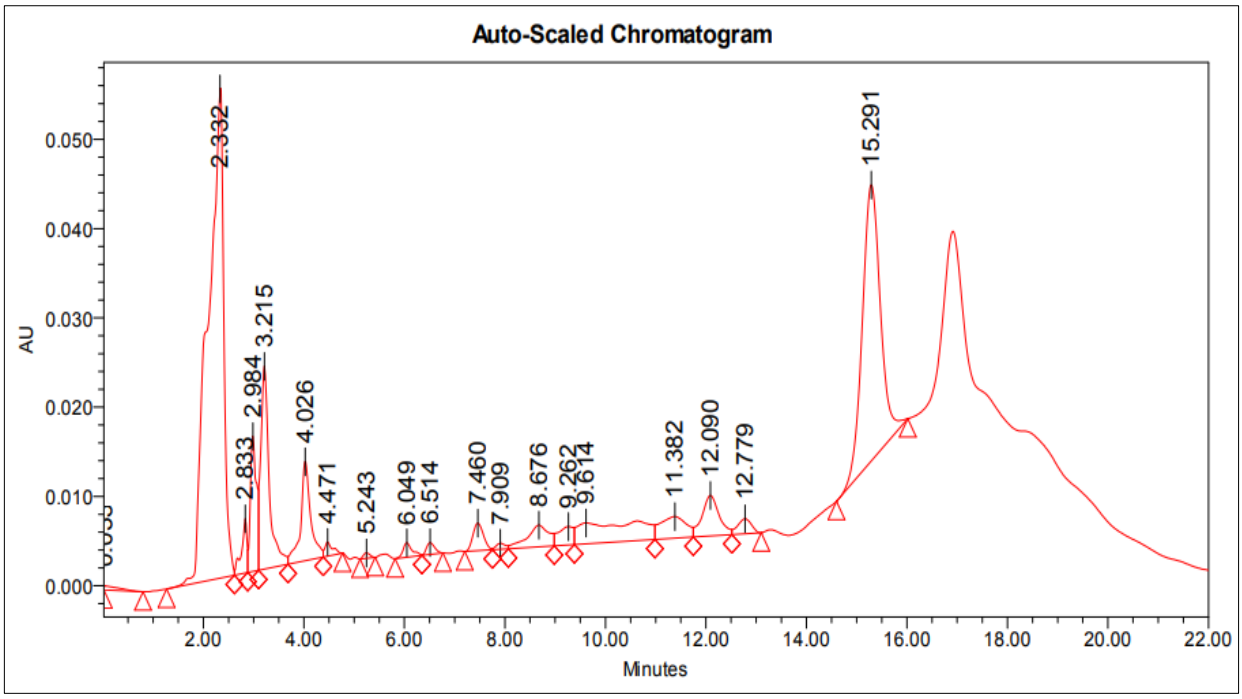

Fig 2: HPLC Chromatogram obtained from ethanolic stem extract of *V.subramanii*

Table 2: Peak value of ethanolic stem extract of *V.subramanii*

Peak	Ret. Time	Area	Height	Cons	Mark	Name
1	0.033	9598	469	0.760	M	Unknown
2	2.332	1175430	54979	0.525	M	Unknown
3	2.833	44672	5972	1.071	M	Unknown
4	2.984	128404	15140	1.308	M	Unknown
5	3.215	265921	22702	0.467	M	Unknown
6	4.026	152782	11191	0.694	M	Unknown
7	4.471	17006	1562	31.405	M	Unknown

8	5.243	5342	597	0.557	M	Unknown
9	6.049	19844	1613	3.814	M	Unknown
10	6.514	14841	1315	16.422	M	Unknown
11	7.460	44601	3082	1.335	M	Unknown
12	7.909	8546	668	37.121	M	Unknown
13	8.676	72809	2431	0.494	M	Unknown
14	9.262	42697	2055	1.094	M	Unknown
15	9.614	189200	2336	0.675	M	Unknown
16	11.382	87064	2430	2.157	M	Unknown
17	12.090	105856	4539	0.557	M	Unknown
18	12.779	31763	1670	0.467	M	Unknown
19	15.291	771484	30945	0.525	M	Unknown
Total		3187860	165696			

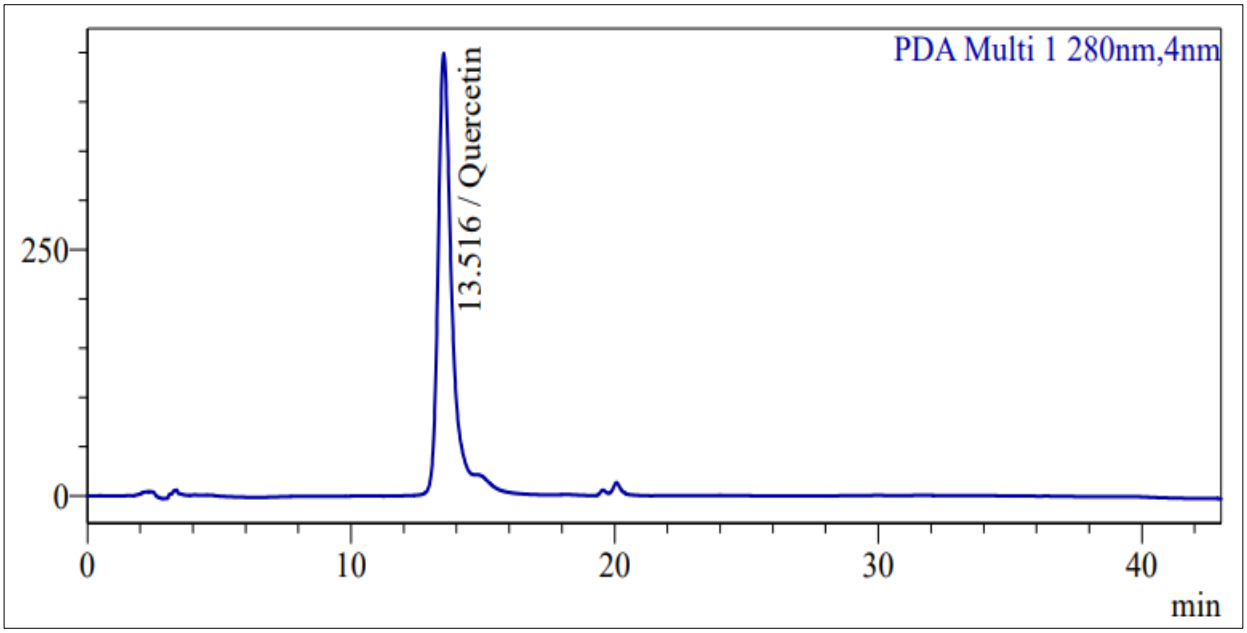

Fig 3: HPLC Chromatogram obtained from a standard quercetin

Table 3: Peak value of quercetin

Peak	Ret. Time	Area	Height	Cons	Unit	Mark	Name
1	13.516	16898105	449889	0.000	mg/L	M	Quercetin
Total		16898105	449889				

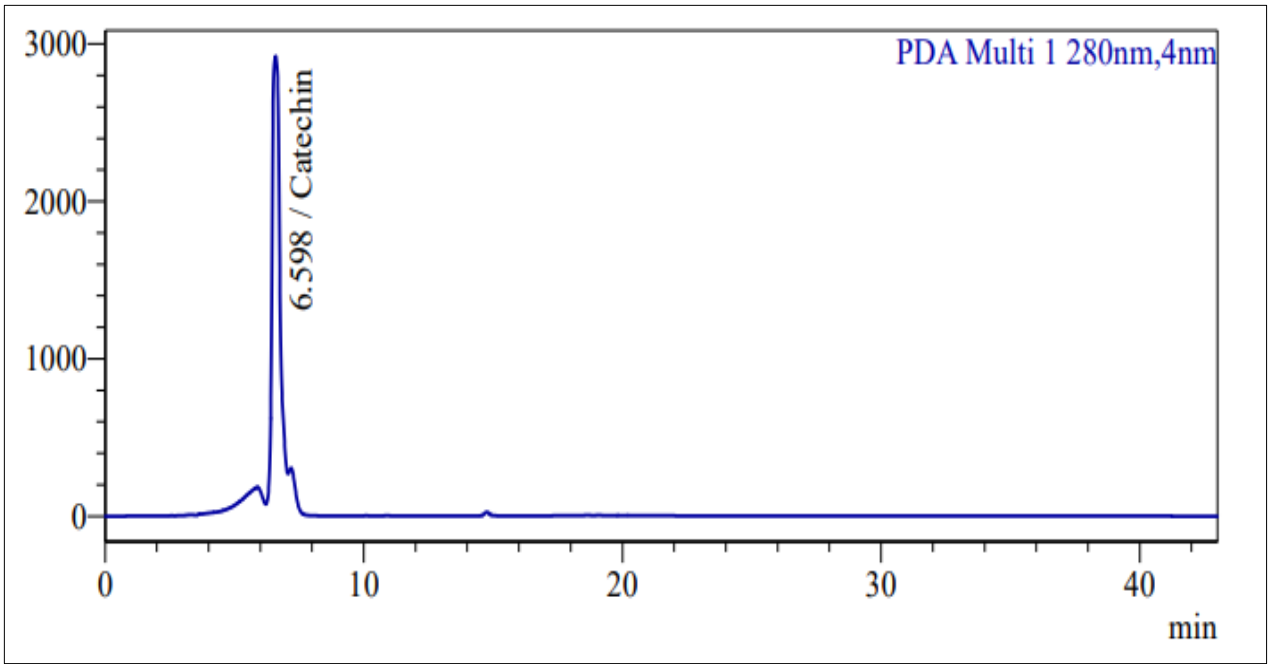

Fig 4: HPLC Chromatogram obtained from a standard catechin

Table 4: Peak value of catechin

Peak	Ret. Time	Area	Height	Cons	Unit	Mark	Name
1	6.598	67426065	2885986	0.000	mg/L	M	Catechin
Total		67426065	2885986				

Qualitative HPLC examination of secondary metabolites revealed that the stem of *V. subramanii* is composed of antioxidant compounds (Figure 2). It was verified by comparing the chromatogram obtained from reference standards Quercetin and catechin (Figure and Table.3,4) were 6,049, 6.514, 11.382, 12.090, 12.779 and 15.291 respectively for the ethanol extract of *V. subramanii* stem, which corresponded well with the standard. The presence of catechins in greater amounts in the chromatographic peaks of quercetin and catechin at 13.516 and 6.598 min, respectively, can be considered as a point of interest in the contribution of plant extracts to antiproliferative activity. At RP-18e, HPLC test outcome for ethanol extraction of leaves of *V. subramanii*. Chromatograms obtained at different storage stages highlight the presence of different botanical components. There are 19 peaks indicating the main components found in the plant *V. subramanii*. In the absence of an autistic criterion for alkaloids, this method produces chromatograms of various compounds found in the ethanol extract of *V. subramanii*. The total UV spectrum of alkaloids is shown in the plant *V. subramanii*, belonging to the parthenolide a family of Sesquiterpenes.

Discussion

Qualitative analysis of phytochemicals from plant samples typically provide valuable compound information for pharmacological and pathological drug development [27]. Extraction of pharmacologically pure active ingredients from plants remains a time consuming and labor-intensive process., there is an urgent need for alternative processes that can eliminate unnecessary separation processes. This makes layer chromatography (TLC) the simplest and most cost-effective method for detecting plant constituents, as it is easy to perform, reproducible and requires minimal equipment [28]. Cardioactive glycosides, sugars, flavonoids and alkaloids were found to be the most abundant secondary metabolites in all species studied. Furthermore, polyphenols have been found in both V. fuscatum and V. parviflorum cultivars. All CH2Cl2, MeOH, CH2Cl2 (1:1) and MeOH isolates [29]. According to recent studies, the *V. subramanii* ethenolic stem extract contains various secondary compounds such as alkaloids, flavonoids and phenols. Due to the presence of different solvent systems, each secondary metabolite behaves differently. This TLC is helpful in understanding solvent polarity. This is important for secondary metabolite detection and compound isolation. This study will support future research on compound separation. In ethanol preparations from *V. subramanii* stem, HPLC analysis showed the presence of flavonoids and phenolic substances such as quercetin and catechins. There are many techniques for measuring phenolic compounds, but HPLC is one of the most important.

In plants, HPLC is one of the widely accepted tools for the qualitative evaluation of plant extracts, derived chemicals and formulations [30]. Quercetin is a well-known natural antioxidant and flavonoid molecule, followed by isoquerbetin, quercitrin, and kaempferol. Apart from being antioxidants, flavonoid compounds play an important role in cell protection. Tannins are high-molecular-weight, polyphenol-rich compounds present in anticarcinogenic, cardioprotective, and other layers of plant tissue. Tannin extracts contain high levels of epicatechin and catechins, which have antioxidant, antitumor, and antibacterial properties [31]. HPLC investigation found that the original sample contained quercertin and catechins. The significant anticancer effect and activity of *V. subramanii* ethanol stem extracts may be due to the presence of any of these chemicals alone or in the latex that may affect therapeutic efficacy.

Conclusion

Results of the current study showed that secondary compounds are abundant in *V. subramanii* stem. These results indicate that *V. subramanii* may be an important source of natural antioxidants for use as therapeutic agents and to treat degenerative diseases caused by oxidative stress. the developed TLC and HPLC methods assist manufacturers in quality control and standardization of herbal formulations. Developed TLC and HPLC techniques to assist manufacturers in quality control and normalization of herbal products. So these fingerprints help distinguish species from admixtures and serve as biochemical markers for the pharmaceutical industry and systematically for this pharmacologically important plant species. Plant research in action, this study confirms the medicinal use of this kind by rural people in the western region of Tamil Nadu. The Stem of *V. subramanii* can provide lead molecules that can be used as starting points for the synthesis of novel broad-spectrum antibiotics that are used to fight diseases caused by living organisms. Future research will focus on the purification, identification and characterization of the active ingredient.

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