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Chemical profiling of *Thymus vulgaris* L. using HPTLC

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Abstract
Objective: To establish the chemical fingerprint of various secondary metabolites of *Thymus vulgaris* L., a culinary and medicinally important plant.
Methods: Preliminary screening for various secondary metabolites was carried out. HPTLC profiles of various metabolites were done and profiles were developed for authentication.
Result: The HPTLC chromatogram showed the presence of 9 Alkaloids, 11 Phenolic compounds, 11 Flavonoids, 10 Essential oils, 8 Tannins, 15 Triterpenes, 12 Steroids and 14 Saponins.
Conclusion: The HPTLC fingerprint obtained can be used as a biomarker which is used for differentiating the species from the adulterant.

Keywords: *Thymus vulgaris* L. Phytochemical screening, HPTLC, Chemical profile, Lamiaceae

1. Introduction
Medicinal plants are resources of many traditional and modern medicines. Several important drugs used in modern medicine have evolved from medicinal plants. The current scenario exhibits the demand for many plant drugs throughout the world. The quality, efficacy and safety of herbal drugs can be ensured by finding reliable features of the plants especially the phytochemicals. It has been established by WHO that herbal medicines aid the health needs of about 80 percent of the world's population; especially for millions of people in the vast rural areas of developing countries [1]. Meanwhile, the need for scientific investigations on medicinal plants becomes forthcoming. This would ensure their liability and repeatability of pharmacological and clinical research to understand their bio-activity and to enhance the product quality control.
Thymus vulgaris L., commonly called as Thyme, form a huge genus of the *Lamiaceae*, or mint family (formerly called *Labiatae*), is a widely used spice and medicinal plant in pharmaceutical industries. Thyme is the general name for many herb varieties of the *Thymus* species, all of which are native to Europe and Asia. Among different species of *Thymus*, *T. vulgaris* L. is the one, widely used than other species, in therapeutic dosage forms. *T. vulgaris* is cultivated in many countries by most people especially in rural areas depending on herbal medicines to treat many diseases including inflammation-related ailments such as rheumatism, muscle swelling, insect bites, pains, etc. It is also used in modern medicine, especially the essential oils of Thyme have shown anti-inflammatory, antioxidant, antibacterial and antifungal properties [2].
Thymes are small woody sub shrubs and perennials, many-branched and aromatic, with small leaves and two-lipped flowers. The leaves are small, opposite, sessile, and gray-green with slightly rolled edges. The small, blue-purple flowers are two-lipped and grow in dense, whorled clusters, blooming from May to September. The aroma comes from essential oils glands in the leaves, calyces and corollas (flowering parts). They are upright growing to 18 inches. Thyme is often a key ingredient in poultry seasoning for chicken and turkey as well as other dishes with fish and meats. It is commonly found in sauces, marinades, sausages and soups as a flavouring ingredient [3].
T. vulgaris is used more in pharmaceutical dosage forms because it contains more essential oils with high amount of Thymol which exhibits considerable antitussive, spasmolytic and expectorant activities [4]. Its antiseptic property is estimated to be 25 times more effective than phenol, with less toxicity [5]. Generally, they contain flavonoids and phenolic compounds such as rosmarinic acid which may have anti-edemic and macrophage-inhibiting effects [6]. An ointment containing Thyme was useful to reduce swelling, warts, sciatica and spleen pains. Bees are also attracted to the plant, as a result, it is used as a flavouring agent in honey [7]. Thyme essential oil constitutes raw material in perfumery and cosmetics due to a special and characteristic aroma [8]. Thymol and carvacrol are the most important constituents of volatile oils of the species [9].

Other chemical constituents of the *T. vulgaris* include caffeic acid, triterpenoids, long-chain saturated hydrocarbons and aliphatic aldehydes [10].

Considering the fact that *T. vulgaris* L. is more expensive, some herbal industries tend to use other species of *Thymus* having different components, for many drugs. So, it is crucial to develop a suitable and reliable identification method to confirm the quality of extracts and herbal drugs. Separation and detection of different constituents in plants are always complicated. Nowadays, HPTLC remains the immediate, simplest, reliable analytical tool to check the presence and the identity of compounds. Development of chemical fingerprints using HPTLC is an effective tool for the separation of chemical compounds leading to the identity of plants. It is efficient and economic for the analysis of broad number of compounds [11]. It has the potential to determine authenticity and reliability of chemical constituent of herbal drug and formulation.

The study on development of individual secondary metabolites of leaves of *Thymus vulgaris* L. by HPTLC has not yet been reported. With this background, an attempt has been made to establish a profile of the various secondary metabolites of *T. vulgaris* L. using HPTLC.

2. Material and Methods

2.1 Collection & Identification

The leaves of the plant *Thymus vulgaris* L. were collected in the month of April-May from Ooty, Tamil Nadu, India. They were identified and authenticated at herbarium of New Udaya Pharmacy & Ayurvedic Laboratories, Cochin, Kerala, India. (Accession numbers N/PG/073). The leaves were air dried, pulverized and used for analysis.

2.2 Extraction

The powdered samples were subjected to extraction in 200 ml methanol for 8-12 hours using the Soxhlet apparatus. The filtered extract was then concentrated and redissolved in Methanol for developing HPTLC fingerprint profile of various secondary metabolites.

2.3 Preliminary Phytochemical Analysis

Qualitative phytochemical analysis of crude extract was determined as follows:

Alkaloids-Alkaloids (0.5g of extract + 5ml of 1 % aqueous hydrochloric acid+ water bath. 1ml filtrate + 6 drops of Mayers/Wagners reagent/ Dragendorff's reagent. Creamish brownish red and orange precipitates indicated presence of respective alkaloids [12]. Phenolic compounds - plant extract treated with 2% ferric chloride solution. A blue-green or black colouration indicated the presence of phenols [13]. Flavonoids - Flavonoids (Shinoda test). A 2 ml filtrate + conc. HCl + magnesium ribbon. Pink or red colour indicated the presence of flavonoids [14]. Cardiac glycosides - Cardiac glycosides (Keller Killiani test), blue colour indicated the presence of cardiac glycosides [15]. Tannins- Tannins (200mg powder in 10ml distilled water, filter). A 2 ml filtrate + 2ml FeCl3 blue black precipitate indicated the presence of tannins. Triterpenoids - (Libermann-Burchard reaction) pinkish red colour indicated presence of terpenoids [16]. Saponins - Saponins (frothing test: 0.5ml filterate+5ml distilled water), frothing persistent meant saponins present. Steroids - Steroids (Libermann-Burchard reaction) blue green ring indicated presence of Steroids.

2.4 HPTLC Fingerprinting

All the chemicals, including solvents were of analytical grade. The HPTLC plates Si60F254 (10X 10) (Merck, Germany) of 0.2mm thickness were used as stationary phase. 10 µL of each extract were applied on the plate of 10 X 10 cm as bands of 10 mm width of each with the help of CAMAG linomat V sample applicator. The plates were developed in a CAMAG twin- trough chamber previously equilibrated with a mobile phase for 20 minutes. Different solvent systems were used to develop HPTLC fingerprint profile for different secondary metabolite groups separately [17]. Each plate were developed up to 80mm, air dried and the plate was kept in photo documentation chamber (Camag Reprostar TLC Scanner 3) and captured the images under White light, UV light at 254 and 366 nm. Densitometric scanning operated by WINCATS software. The chromatograms were recorded and then the plates were derivatized with respective chemical reagents and heated at 105 ^{0}C on hot plate till the development of colour of bands and observed under white light. The colour of recorded bands and Rf values were recorded.

3. Results

Phytochemical analysis revealed the presence of Alkaloids, Phenolic compounds, Steroids,
Flavonoids, Tannins, Triterpenoids, and Saponins (Table 1). HPTLC screening showed the presence of Essential oils in addition.

Table 1: Preliminary Screening of Secondary metabolites from Methanolic extract of *Thymus vulgaris* L. (Leaves)

Sl. No.	Secondary metabolites	Test	Presence
1.	Alkaloids	Mayer's test	+
2.	Phenolic compounds	Ferrric chloride	+
3.	Steroids	Libermann-Burchard	+
4.	Flavonoids	Shinoda test	+
5.	Cardiac glycosides	Keller Killiani test	-
6.	Tannins	FeCl3 test	+
7.	Triterpenoids	Libermann-Burchard	+
8.	Saponins	Frothing test	+

HPTLC

Alkaloids

The HPTLC chromatogram for Alkaloids was best observed at 254nm & 366nm before derivatization. 9 bands of Alkaloids were seen to be separated before derivatization at 366 nm. The compounds separated were seen at Rf = 0.00,0.08, 0.19, 0.38, 0.48, 0.58, 0.71, 0.86 and 0.91. Best solvent system to observe the above separation is Toluene: Methanol: Diethyl amine (8:1:1). Dragendorff's reagent was used for derivatization (Plate 1).

Phenolic compounds

The HPTLC chromatogram can be best observed under 254nm & 366nm before and after derivatization. 11 bands were seen to be separated before derivatization at 366nm. The major compounds separated were seen at Rf = 0.00, 0.04, 0.16, 0.26, 0.37, 0.43, 0.50, 0.61, 0.66, 0.79 and 0.82. Best solvent system to observe the above separation is: THF: Toluene: Formic acid: Water (16:8:2:1). Fast Blue Salt B used as derivatizing agent (Plate 2).

Flavonoids

The HPTLC chromatogram can be best observed under fluorescence 254nm &366 nm before and after derivatization.

11 bands of Flavonoids are seen to be separated before and after derivatization at 366 nm. The major compounds separated were seen at Rf = 0.00, 0.11, 0.15, 0.23, 0.27, 0.31, 0.38, 0.50, 0.66, 0.76 & 0.85. Best solvent system to observe the above separation is: Toluene: Ethyl acetate: Formic acid (7:3:0.1). NP/PEG Reagent was used for derivatization (Plate 3).

Essential oils

The HPTLC chromatogram for Essential oils can be observed at 254 nm and 366nm before and after derivatization. 10 bands of Essential oils are seen to be separated before derivatization at 366 nm. The major compounds separated were seen at Rf = 0.03, 0.12, 0.14, 0.23, 0.30, 0.51, 0.60, 0.72 & 0.80. Solvent system: Toluene: Ethyl acetate (8.5:1.5). Derivatization using Anisaldehyde sulphuric acid (Plate 4).

Tannins

The HPTLC chromatogram for Tannins was best observed at 366 nm before and after derivatization.8 bands of Tannins were seen to be separated. The major compounds separated were seen at Rf = 0.00, 0.07, 0.12, 0.17, 0.22, 0.32, 0.58 & 0.75. Best Solvent system: Ethyl acetate: Acetic acid: Ether: Hexane (4:2:2:2). Fast Blue Salt B was used as derivatizing agent (Plate 5).

Triterpenes

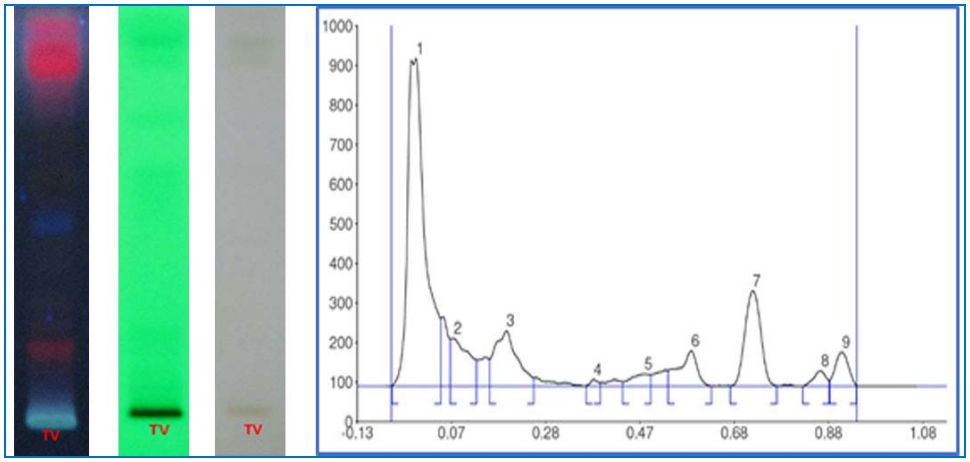
The HPTLC chromatogram can be best observed at wavelength 254 nm and 366 nm before and after derivatization. A good separation of compounds were observed. 15 major compounds were separated and seen at Rf = 0.00, 0.08, 0.12, 0.17,0.23, 0.25, 0.29, 0.33, 0.41, 0.45, 0.47, 0.51, 0.68, 0.77 & 0.82. Toluene: Chloroform: Ethanol (4:4:1) was the first solvent system to observe the above separation. Derivatizing reagent used was Anisaldehyde sulphuric acid (Plate 6).

Steroids

Steroids can be observed at wavelength 254 nm and 366 nm before and after derivatization. 12 bands of Steroids are seen to be separated before derivatization at 366 nm. The major compounds separated were seen at Rf = 0.00, 0.06, 0.19, 0.24, 0.30, 0.37, 0.49, 0.59, 0.62, 0.68, 0.75 & 0.84. Solvent system: Toluene: Methanol: Acetone (6:2:2). Anisaldehyde sulphuric acid was the derivatizing agent (Plate 7).

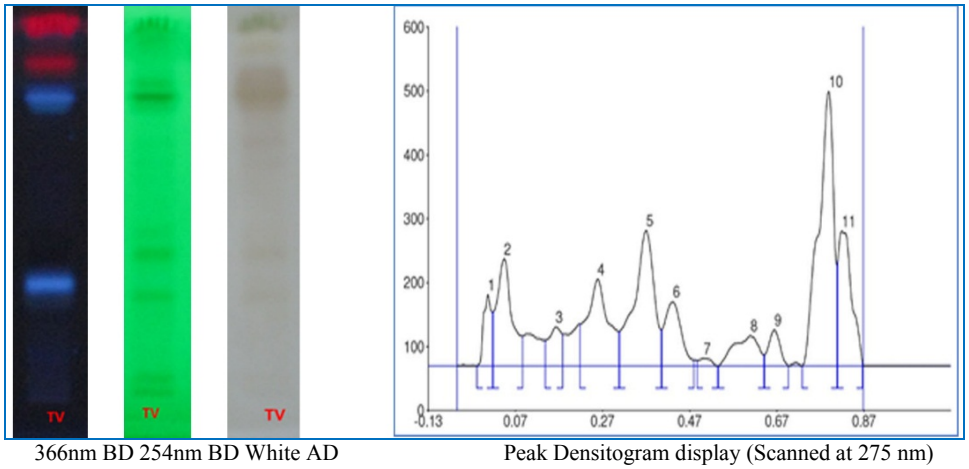
Saponins

The HPTLC chromatogram can be best observed at wavelength 254 nm and 366 nm before and after derivatization. 14 major compounds were seen to be separated at Rf = 0.00, 0.07, 0.13, 0.21, 0.25, 0.30, 0.36, 0.40, 0.48, 0.52, 0.62, 0.70, 0.77& 0.83. Best solvent system to observe the above separation is Toluene: Chloroform: Ethanol (4:4:1). Derivatizing agent used was Anisaldehyde sulphuric acid (Plate 8).



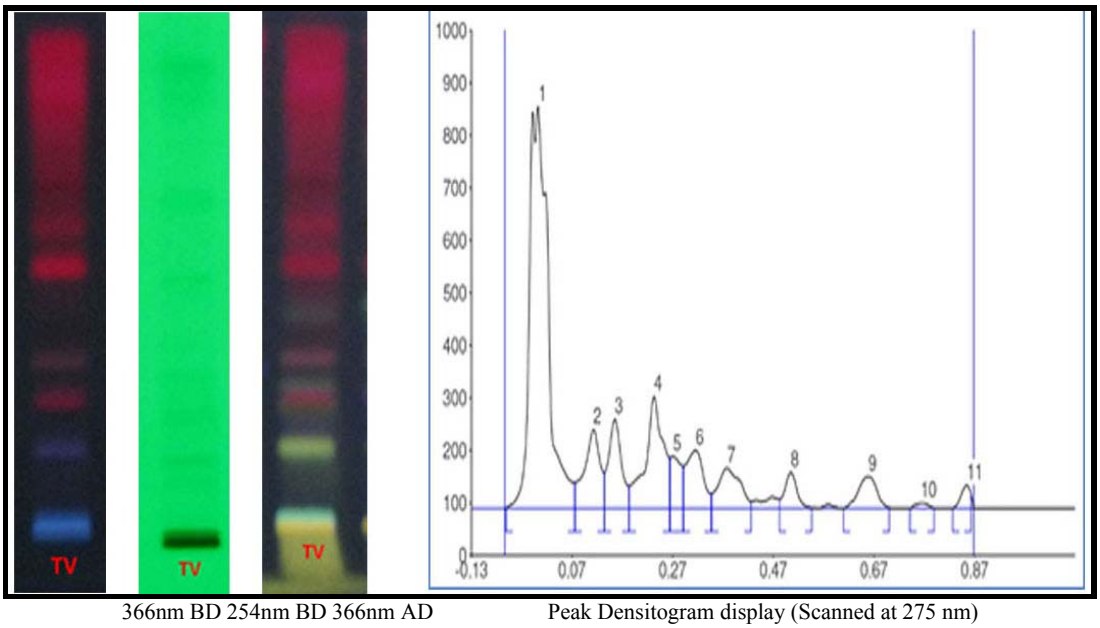
366nm BD 254nm BD White AD Peak Densitogram display (Scanned at 275 nm)

Plate 1: HPTLC fingerprint of *Thymus vulgaris* L. for Alkaloids



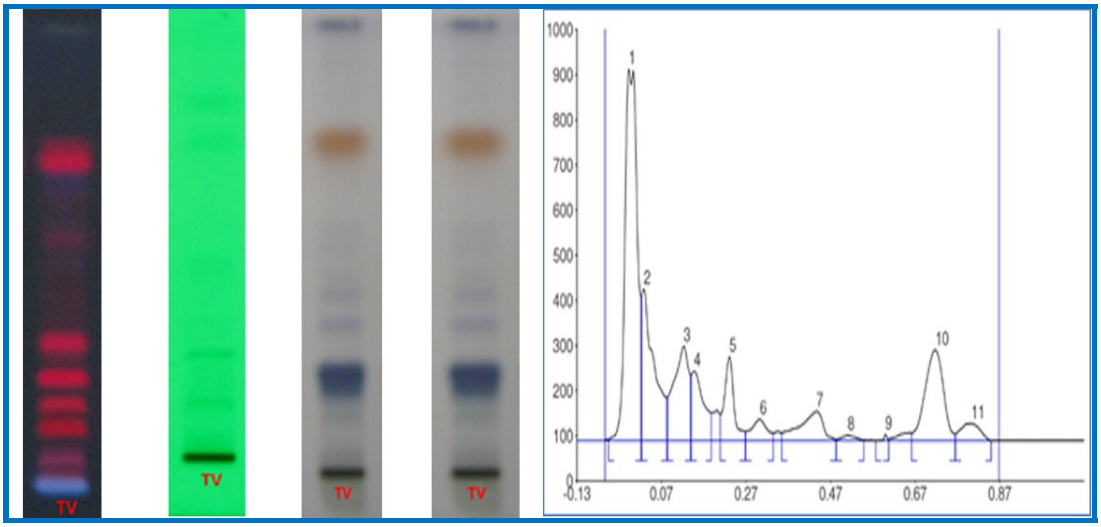
366nm BD 254nm BD White AD Peak Densitogram display (Scanned at 275 nm)

Plate 2: HPTLC fingerprint of *Thymus vulgaris* L. for Phenolic compounds



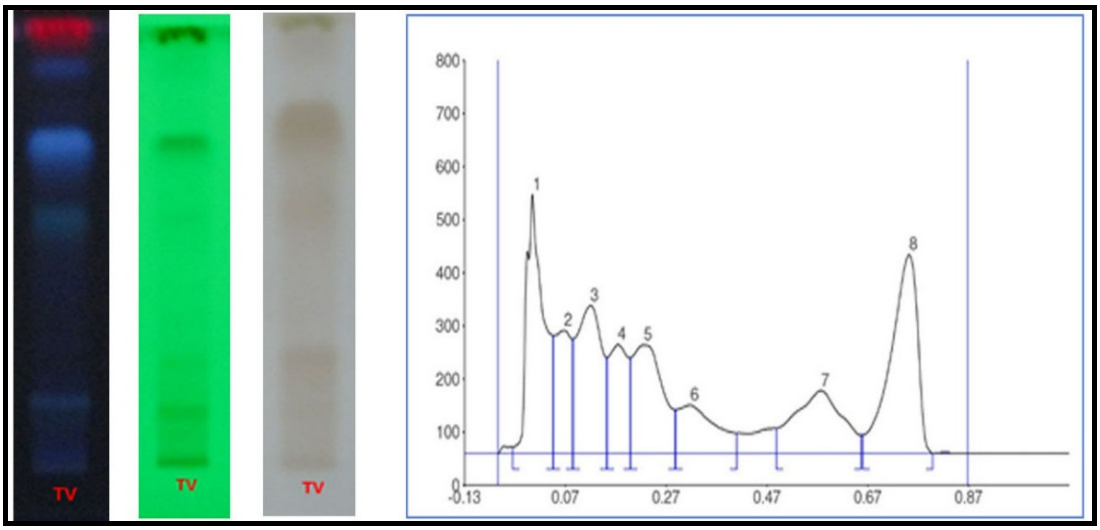
366nm BD 254nm BD 366nm AD Peak Densitogram display (Scanned at 275 nm)

Plate 3: HPTLC fingerprint of *Thymus vulgaris* L. for Flavonoids



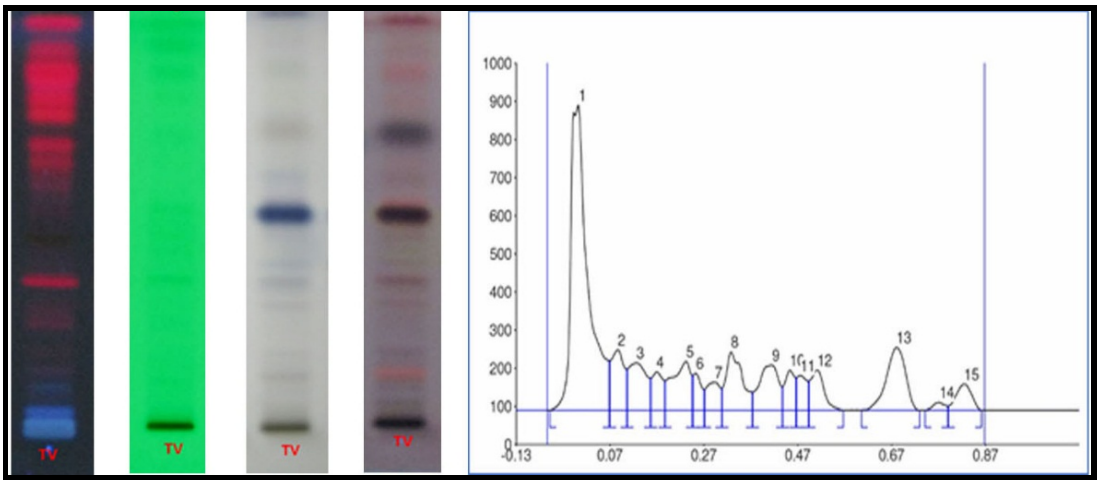
366nm BD 254nm BD White AD 366nm AD Peak Densitogram display (Scanned at 275 nm)

Plate 4: HPTLC fingerprint of *Thymus vulgaris* L. for Essential oils



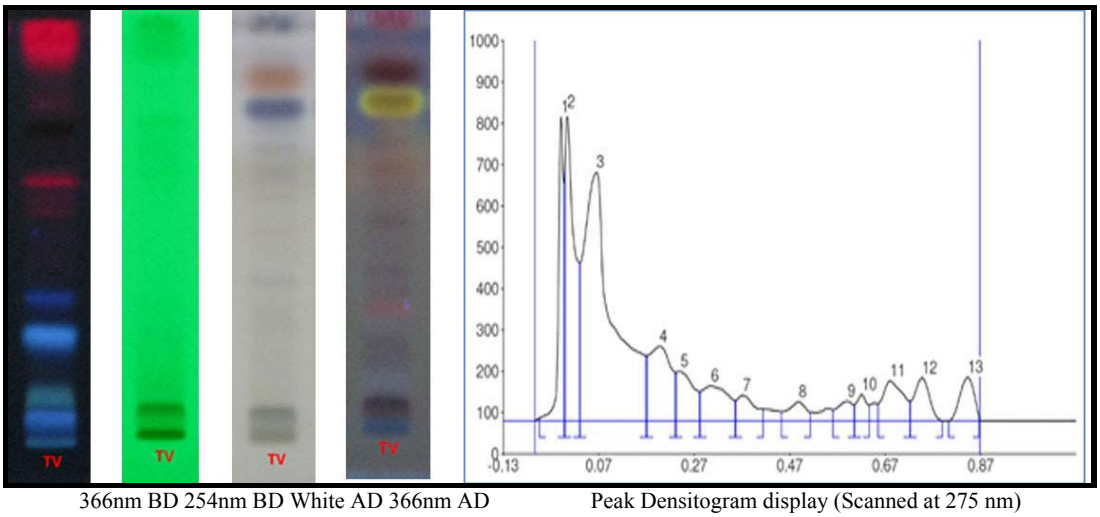
366nm BD 254nm BD White AD Peak Densitogram display (Scanned at 275 nm)

Plate 5: HPTLC fingerprint of *Thymus vulgaris* L. for Tannins



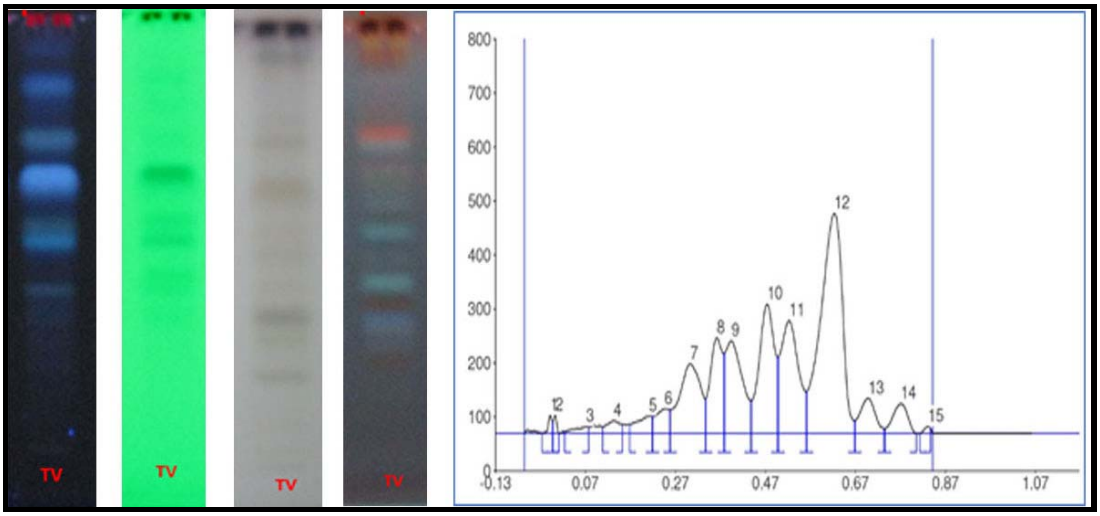
366nm BD 254nm BD White AD 366nm AD Peak Densitogram display (Scanned at 275 nm)

Plate 6: HPTLC fingerprint of *Thymus vulgaris* L. for Triterpenes



366nm BD 254nm BD White AD 366nm AD Peak Densitogram display (Scanned at 275 nm)

Plate 7: HPTLC fingerprint of *Thymus vulgaris* L. for Steroids



366nm BD 254nm BD White AD 366nm AD Peak Densitogram display (Scanned at 275 nm)

Plate 8: HPTLC fingerprint of *Thymus vulgaris* L. for Saponins

4. Discussion

Medicinal plants are resources of traditional medicines and many of the modern medicines are produced indirectly from plants. Nature produces a tremendous array of secondary metabolites or natural products from plants [18, 19]. Plants and their metabolites constituents have a long history of use in modern 'western medicine' and in certain systems of traditional medicine and are the sources of important drugs [20]. Natural products are the main sources of bioactive molecules and have played a major role in leading the

discovery of compounds for the development of drugs for treatment of human diseases [21]. These compounds have important ecological functions providing defense mechanisms. Medicinal plants are having chemical compounds used as antimicrobial and antioxidant agents for treating many diseases. Humans exploit plants as a source of drugs, flavouring agents, fragrances and for a wide range of other applications. A vast array of secondary metabolites was found to be present in the plant under study. The Methanolic extract of the leaves of *Thymus vulgaris* L. showed the presence of 9 Alkaloids, 11 Phenolic compounds, 11 Flavonoids, 10 Essential Oils, 8 Tannins, 15 Triterpenoids, 12 Steroids and 14 Saponins.

Thymus vulgaris L. possesses antispasmodic, antiseptic, expectorant, carminative and anti-oxidative properties [22, 23]. Pure, isolated plant alkaloids and their synthetic derivatives are used as basic medicinal agents for their analgesic, anti spasmodiac and bactericidal effects [24]. *T. vulgaris* L. showed good separation of Phenolic compounds, Flavonoids and Triterpenoid compounds. The determination of total phenols content is of great importance in plants. It intervenes in the growth process, protection of plants against insect pests. In addition, they contribute to give colour and flavour specific to fruit and vegetables [25, 26]. A good separation of Flavonoids has been observed. Thyme is a rich source of flavonoid and phenolic antioxidants such as *zea-xanthin*, *pigenin, lutein, luteolin* and *thymonin* [27].

A good separation of Essential oils has been observed. 10 bands of essential oils were seen to be separated. The Essential oil of Thyme has also been reported to be effective in food preservatives, antioxidants, antimicrobials, breath-freshening dentifrices, medical disinfectants, etc. [28]. According to a previous study, the percentage of Tannins, Saponins and Volatile oils was found to be (9.2%, 23.1%, 50.7%) respectively [29]. Saponins are other type bioactive chemical constituents which are involved in plant disease resistance because of their antimicrobial activity [30]. Tannins are phenolic compound and their derivatives are also considered as primary antioxidants or free radical scavengers [31].

T. vulgaris L. showed the maximum number of bands in Triterpenes. Terpenes have also shown antimicrobial activities. This is important due to the increase in antibiotic resistant bacteria, which is occurring globally and at an alarming rate [32-34]. Triterpenoids constitute a wide, biologically interesting group of terpenoids and include a large structural diversity of secondary metabolites identified from terrestrial and marine living organisms. Plant steroids possess many interesting medicinal, pharmaceutical and agrochemical activities like anti-tumor, immunosuppressive, hepatoprotective, antibacterial, plant growth hormone regulator, sex hormone, antihelminthic, cytotoxic and cardiotonic activity [35].

5. Conclusion

Quality control and Standardisation of the plant materials used as drug or nutraceuticals, is the need of the day. Therefore, the availability of analytical tools to assess the complexity of natural products mixtures is very crucial. The HPTLC fingerprint approach is extremely effective in the identification of the plant used especially in mixtures. A fingerprint is the individual chromatographic track representing, as near as possible, a mixture of organic substances. By the fingerprint approach, it is possible to attain a proper identification of the plant material. Variations in HPTLC tracks of the same species are mainly quantitative, not qualitative.

HPTLC analysis revealed a better separation of individual secondary metabolites. The plant under study can be used to discover bioactive products which may lead to the development of the new pharmaceuticals. Bioactivity guided fractionation can lead to the isolation of active components of this plant. The HPTLC fingerprint of secondary metabolites can be used as a 'Biomarker' for the identification of *Thymus vulgaris* L.

6. Acknowledgement

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7. Conflict of interest statement

We declare that we have no conflict of interest.

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