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Total Flavonoids, Total Phenolics and Targeted HPTLC Profile for Quantification of Quercetin in Wedelia chinensis and Cassytha filiformis

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ABSTRACT

This study quantitatively determined the total phenolic and flavonoid contents in hydroalcoholic extracts of *Wedelia chinensis* and *Cassytha filiformis* using a spectrophotometric method. We also performed an HPTLC profile for both plant extracts to determine the quantity of quercetin. A sensitive and reliable high-performance thin-layer chromatographic method has been developed to quantify quercetin in dried whole plant powder. *Wedelia chinensis* and *Cassytha filiformis* hydroalcoholic extract was chromatographed on silica gel 60 F254 plates. The linear ascending development was carried out in a twin trough glass chamber (CAMAG, Muttenz, Switzerland) with Toluene: Acetone: Formic Acid (4.5:4.5:1) (v/v/v), as mobile phase. Total phenolic content was found to be (155 mg of GAE/g of crude extract) in the *Wedelia chinensis*, and the lowest was in the *Cassytha filiformis* extract (52.01 mg of GAE/g). *Wedelia chinensis* also had the highest total flavonoid content (72.3 ± 0.233 mgQE/g), while the lowest was in the *Cassytha filiformis* (3.19± 0.256 mgQE/g). In this research, the Rf values of quercetin were found to be 0.75 and 0.76 in *Wedelia chinensis* and *Cassytha filiformis*, respectively.

Keywords: *Hydroalcoholic, Phenolics, Flavonoids, Quercetin, HPTLC*

1. INTRODUCTION

India's diverse cultural traditions have long been associated with using herbal plants to cure various diseases. This knowledge, found in thousands of medical theories and manuscripts, has led to extensively using substances with therapeutic potential to treat multiple disease conditions. Herbs, readily available to humans, have been thoroughly explored for their medicinal properties. While products of primary metabolisms are vital for growth and development, secondary metabolites like alkaloids, phenolics, steroids, and terpenoids have therapeutic action in toxicological, pharmacological, and ecological applications for diagnosis, prevention, and treatment. Among the medicinal plants used in Ayurvedic preparations, some have been thoroughly investigated for their therapeutic action, while others hold potential for future exploration.[1,2]

Wedelia chinensis Merrill is a perennial herb of the family Asteraceae, commonly known as “Pilabhamgara” or “Bhringraj” in Hindi, Wedelia in Chinese and “Manjal karisalanganni” in Tamil.[3] Traditionally, the fruits, leaves and stems are used in childbirth and in treating bites and stings, fever and infection. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds. The leaves treat kidney dysfunction, cold, wounds and amenorrhea.[4] The leaves are also for dyeing hair and for promoting their growth. The tonic of the leaves is used for cough and cephalalgia. Decoction of the plant is used in menorrhagia and skin diseases [5,6]

Cassytha filiformis is commonly called Love Vine in English and is a parasite plant that is distributed in many parts of the world, including Central and South America, Africa, Asia and the Pacific islands, mostly in coastal regions.[7] The plant belongs to the Lauraceae family, and the genus Cassytha has over 2500 species distributed throughout tropical to subtropical

areas.[8] This parasitic plant's main host trees are Mango, Citrus, Avocado, Nutmeg, Clove, etc. [9] Many pharmacological activities of this plant have been identified so far, i.e. Vasorelaxant, Anti-Diabetic, Anti-piratic, Anti-inflammatory, Anti-typomania, etc.[10,11]

The phenolic compounds present in the plant were thought to be responsible for its anti- oxidant properties [12,13]. The main objective of this study was to evaluate total phenolic content, total flavonoid, and optimise the quantification of quercetin present in hydroalcoholic extracts of *Wedelia chinensis* and *Cassytha filiformis* by the HPTLC method to provide beneficial information regarding the standardisation according to WHO guidelines. Quick and accurate assessment of phenolic acids and flavonoids is crucial because of their inherent structural diversity for health and dietary significance.[14]

Today's high-performance thin-layer chromatography (HPTLC) technique is incorporated into the pharmacopeial analysis. High-performance thin-layer chromatography (HPTLC)- based methods are a good alternative, as they are being explored as an essential tool in routine drug analysis. HPTLC's primary advantage is its ability to analyse several samples simultaneously using a small quantity of mobile phase. HPTLC is increasingly used as a standard analytical technique for qualitative and quantitative determinations.[15] The results could be handled in automated and manual ways, such as with peak profiles from an image, densitogram, or chromatogram. [16] Due to its benefits of low operating costs, high sample throughput, simplicity, speed, minimal sample cleanup requirements, reproducibility, accuracy, and dependability,

2. METHODOLOGY

Plant Material Collection and Pre-Treatment :

The plants *Wedelia chinensis* and *Cassytha filiformis* were collected from Tirupati, Andhra Pradesh, India, and authenticated by Dr. K. Madhava Chetty, Professor, Department of Botany, Shri Venkateshwara University, Tirupati, India. They were deposited at the college herbarium. (voucher no. 0551)The leaves were washed and shade dried, and the coarse powder was made mechanically for the extraction procedure.

Extraction

50 g of the shade-dried and powdered leaf material was taken for extraction. The crude drug of both plants was subjected to the extraction (cold maceration) method.. The extracts were filtered over Whatman No. 1 filter paper, and the filtrates were concentrated under reduced pressure to a pasty mass for further studies.

The dried whole plant of the plant *W. chinensis* and *Cassytha filiformis* were collected and pulverised through a mechanical grinder. Then, the powder material was dried at room temperature in the shade. The powder material (50 g) was defatted with petroleum ether and then macerated with hydro-alcohol (30:70) for 24 hrs. After that, the extract was concentrated, weighed, and the percentage yield was calculated on an air-dried weight basis. The obtained extract was stored at 4°C till further use [17]

Preparation of standard solutions, buffer, reagent and extract

Gallic acid solution (100 µg/ml): 10 mg of gallic acid was dissolved in 100 ml of distilled water in a volumetric flask.[18]

Quercetin solution (1000 µg/ml): A 1000 µg/ml stock solution was prepared by dissolving 100 mg of quercetin in 100 ml of absolute alcohol. [19]

Determination of total phenol content

Folin Ciocalteu's method was used to estimate total phenolic content. 1 ml of aliquots and standard gallic acid (100, 200, 300, 400, 500 µg/ml) was positioned into the test tubes, and 9 ml of distilled water and 1 ml of Folin Ciocalteu's reagent was mixed and shaken. After 5 minutes, 10 ml of 20 % sodium carbonate was added, and the volume was made up to 25 ml with distilled water.

It was allowed to incubate for 2 hours at room temperature. Intense blue color was developed. After incubation, absorbance was measured at 760 nm using a spectrophotometer with a UV-Visible Shimadzu UV-1800 instrument. The extracts were performed in triplicates. The blank was performed using a reagent blank with solvent. Gallic acid was used as standard. The calibration curve was plotted using standard gallic acid. The data for total phenolic contents of hydroalcoholic extract of both plants, *Wedelia chinensis* and *Cassytha filiformis* were expressed as mg of gallic acid equivalent weight (GAE)/ 100 g of dry mass. [18,20]

Determination of total flavonoid content

Total flavnoid content was measured with the aluminium chloride colourimetric assay. 1ml of aliquots and 1ml standard quercetin solution (100, 200, 300, 400, 500 µg/ml) in ethanol was positioned into test tubes and 4ml of distilled water and 0.3 ml of 5 % sodium nitrite solution was added into each. After 5 minutes, 0.3 ml of 10 % aluminium chloride was added. At the 6th minute, 2 ml of 1 M sodium hydroxide was added. Finally, the volume was made up to 10 ml with distilled water and mixed well. An orange-yellowish colour was developed. The absorbance was measured at 510 nm using a spectrophotometer with a UV-visible Shimadzu instrument. The blank was performed using distilled water. Quercetin was

used as standard. The samples were performed in triplicates. The calibration curve was plotted using standard quercetin. The data of total flavonoids of polyherbal formulation were expressed as mg of quercetin equivalents/ 100 g of dry mass.[19,21,22]

HPTLC Analysis

HPTLC Identification and Quantification of Quercetin in Herbal Extracts

Standard Solution Preparation:

Quercetin Standard: 10 mg of Marker was dissolved in 10 ml of methanol to get 1000 µg/ml solution. 5 ml of this solution was further diluted with methanol to 10 ml to get 500 µg/ml solution

Herbal Extract Solutions : 50 µl of Extract was applied

1. Hydroalcoholic Extract of *Wedelia chinensis*
2. Hydroalcoholic Extract of *Cassytha filiformis*

Instrumentation and Chromatographic conditions[24]

The standards and samples were applied in the form of bands of width of 6 mm with space between bands of 5 mm, with a 100 µL sample syringe (Hamilton, Bonaduz, Switzerland) on precoated silica gel aluminium plate 60 F_{254} (10 cm ×10 cm) with 250 µm thickness (E. MERCK, Darmstadt, Germany) using a CAMAG Linomat 5 sample applicator (Switzerland). The slit dimensions 5 mm × 0.45 mm and scanning speed of 20 mm/sec was employed.

The linear ascending development was carried out in 10 cm×10 cm twin trough glass chamber (CAMAG, Muttenz, Switzerland) using Toluene: Acetone: Formic Acid (4.5:4.5:1) as mobile phase.

The optimized chamber saturation time for the mobile phase was 10 min. The length of the chromatogram run was 8 cm, and development time was approximately 15 min. TLC plates were dried in a current of air with the help of a hair dryer. Densitometric scanning was performed on a CAMAG Thin-Layer Chromatography scanner at 378 nm operated by WINCATS software version 1.4.2.

Chromatographic parameters are summarized in Table.1

Table.1: Chromatographic parameters.

Sr. No.	Parameter	Conditions used for Analysis
1	Stationary phase	TLC aluminum plate pre-coated with silica gel 60 GF_{254}
2.	Mobile phase	Toluene: Acetone: Formic Acid (4.5:4.5:1)
3.	Detection Wavelength	378 nm
4.	Saturation time	10 min
5.	Band width	6 mm

Application of Standard and Sample volumes on TLC Plate:

Track 1 – Standard Quercetin Solution (500 µg/ml) – 2 µl

Track 2 – Standard Quercetin Solution (500 µg/ml) – 4 µl

Track 3 – Standard Quercetin Solution (500 µg/ml) – 6 µl

Track 4 – Standard Quercetin Solution (500 µg/ml) – 8 µl

Track 5 – Standard Quercetin Solution (500 µg/ml) – 10 µl

Track 6 – Standard Quercetin Solution (500 µg/ml) – 12 µl

Track 7 – Wedelia chinensis Extract (50 µl)

Track 8 – Cassytha filiformis Extract (50 µl)

3. RESULT

Determination of total phenolic content

The total phenolic content for aqueous, hydroalcohol and ethanol extracts were estimated by Folin Ciocalteu's method using gallic acid as standard. The reagent is formed from a mixture of phosphotungstic acid and phosphomolybdic acid which after oxidation of the phenols, is reduced to a mixture of blue oxides of tungsten and molybdenum. The blue coloration produced has a maximum absorption in the region of 750 nm and proportional to the total quantity of phenolic compounds originally present. The gallic acid solution of concentration (10-100 ppm) conformed to Beer's Law at 750 nm with a regression co-efficient (R2) = 0.9898. The plot has a slope (m) = 0.0915 and intercept = 0.0869. The equation of standard curve is y = 0.0915x + 0.0869 (Fig. 1).

Fig. 1: Total phenolic content for standard gallic acid.

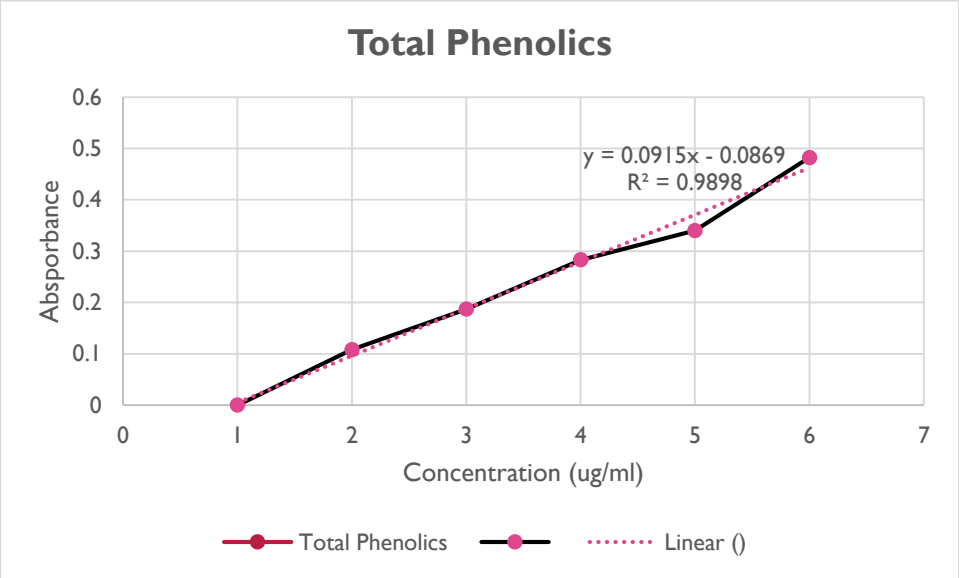


Table 2: Results of total phenolic content for plant extracts

Concentration of extracts	Phenolic content (mg of gallic acid equivalent/ g dry material)
Wedelia chinensis	155.2± 0.233
Cassytha filiformis	52.1 ± 0.256

Values are mean ± S.E.M, n=3

Determination of total flavonoid content

The total flavonoid content for aqueous, hydroalcohol and ethanol extracts were measured with the aluminium chloride colorimetric assay using quercetin as standard. Aluminium chloride forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxide group of flavones and flavonols. In addition it also forms liable complexes with ortho dihydroxide groups in A/B rings of flavonoids. The quercetin solution of concentration (100-1000 ppm) conformed to Beer's Law at 510 nm with a regression co-efficient (R2) = 0.9917. The plot has a slope (m) = 0.0087 and intercept = 0.0642. The equation of standard curve is y = 0.0087x + 0.0642 (Fig. 2).

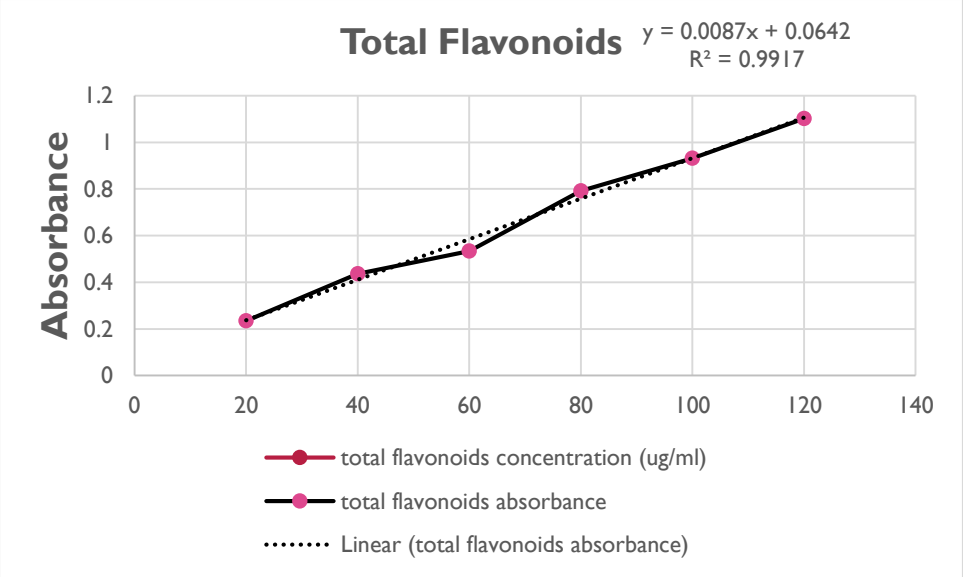


Fig. 2: Total flavonoid content for standard quercetin.

Table.3 : Results of total Flavonoid content for plant extracts

Concentration of extracts	**Flavonoid content (mg of quercetin equivalent/ g dry material)**
Wedelia chinensis	72.3 ± 0.233
Cassytha filiformis	3.19± 0.256

Values are mean ± S.E.M, n=3

3.2 HPTLC

Estimation of quercetin in plant extracts

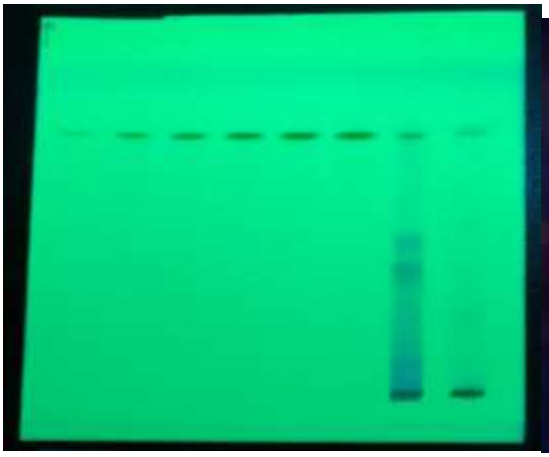
A) Qualitative Analysis:

Extracts found to contain Quercetin by comparing Rf values with standard

Table.4 : Rf value for plant extracts

SN	**Extract**	**Quercetin**	**Rf Value**
1	**Standard Biomarker**		**0.76**
2	**Hydroalcoholic Extract of *Wedelia chinensis***	**+**	**0.75**
3	**Hydroalcoholic Extract of *Cassytha filiformis***	**+**	**0.76**

(+) – Present



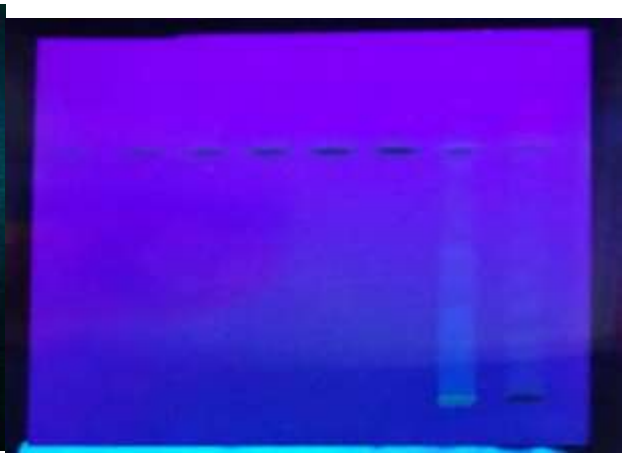
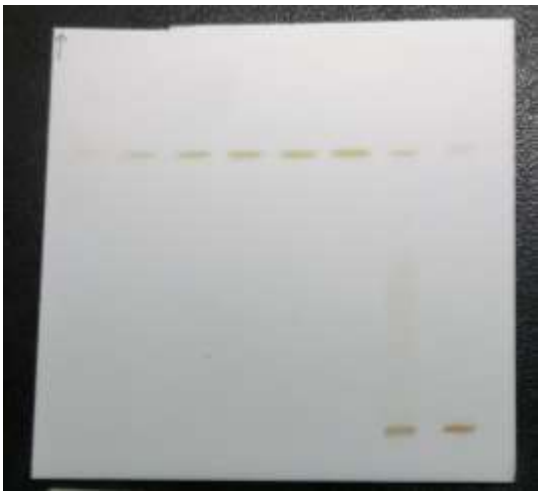


Fig.3: HPTLC plate Seen at 366 nm. **Fig.4: HPTLC plate Seen at 254 nm**



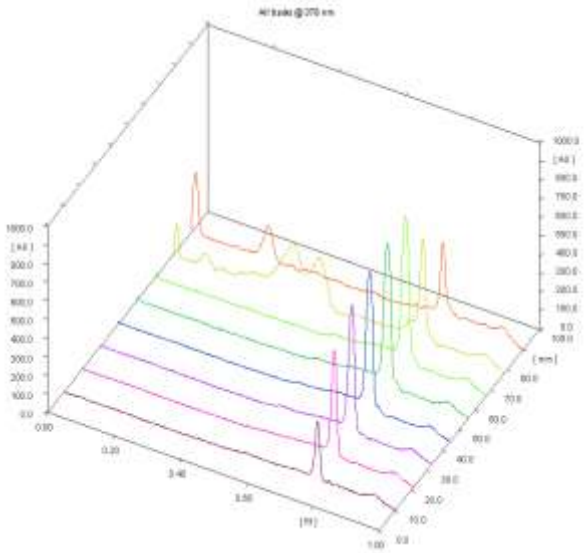
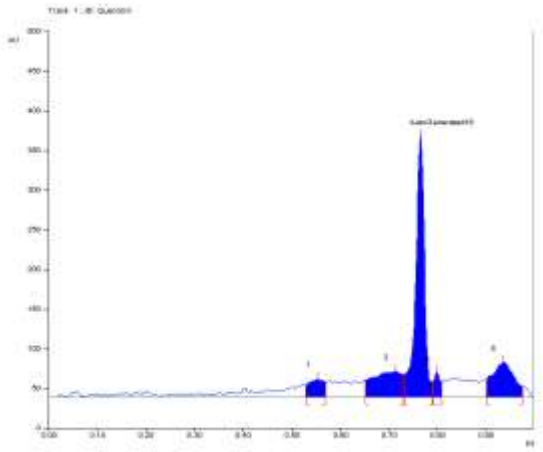
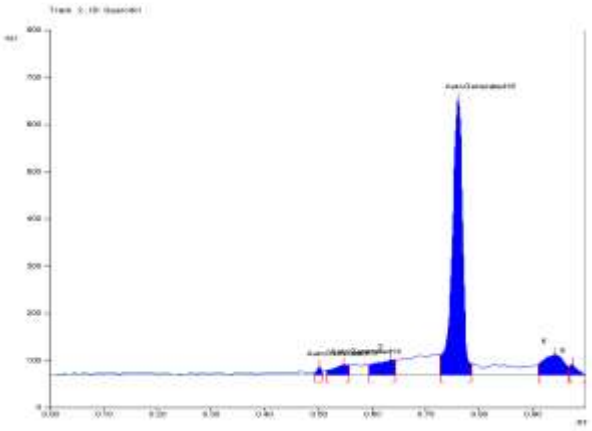
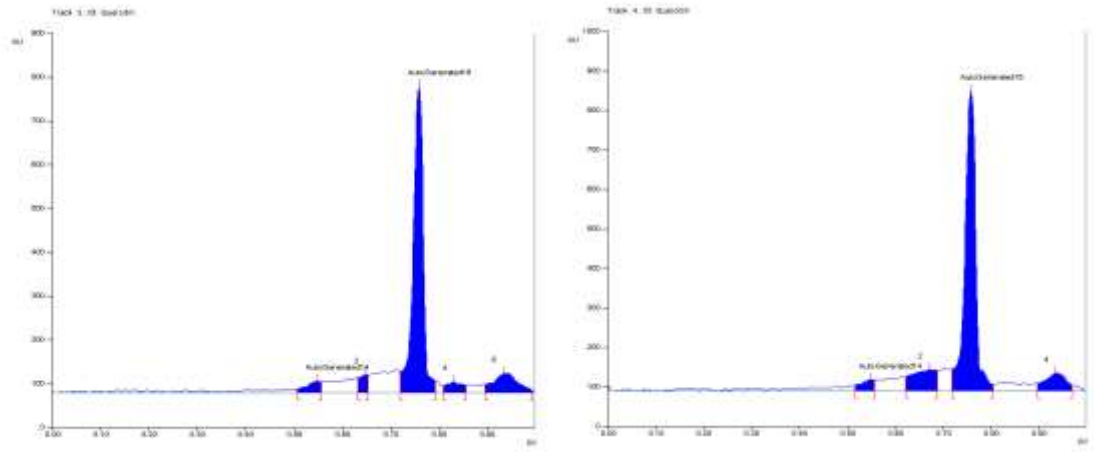


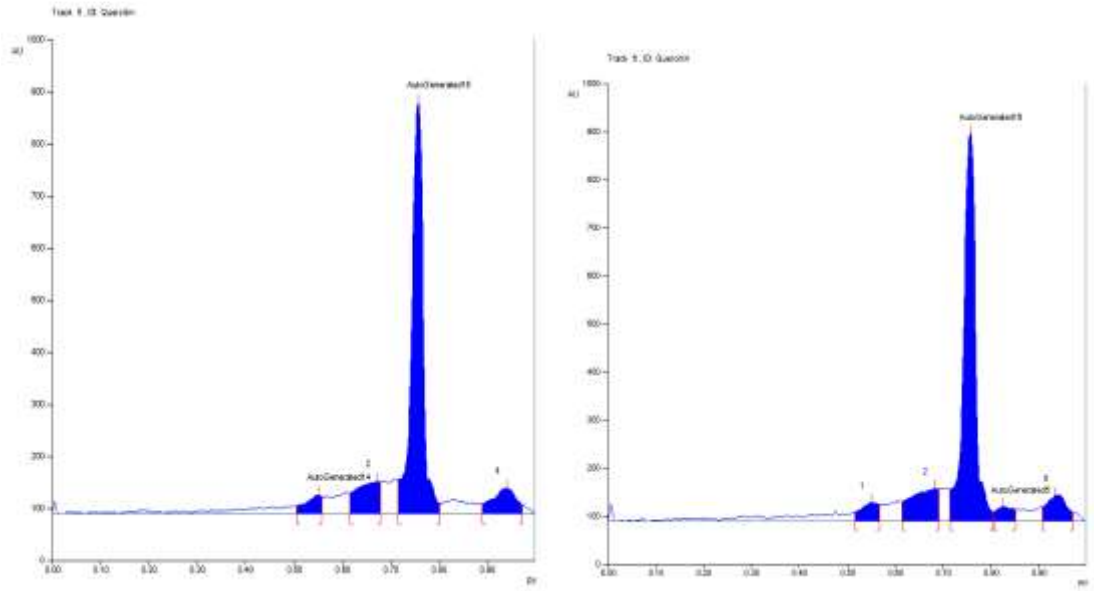
Fig.5: HPTLC plate Seen at Visible Light **Fig.6: 3D Densitogram Plot of HPTLC Development**

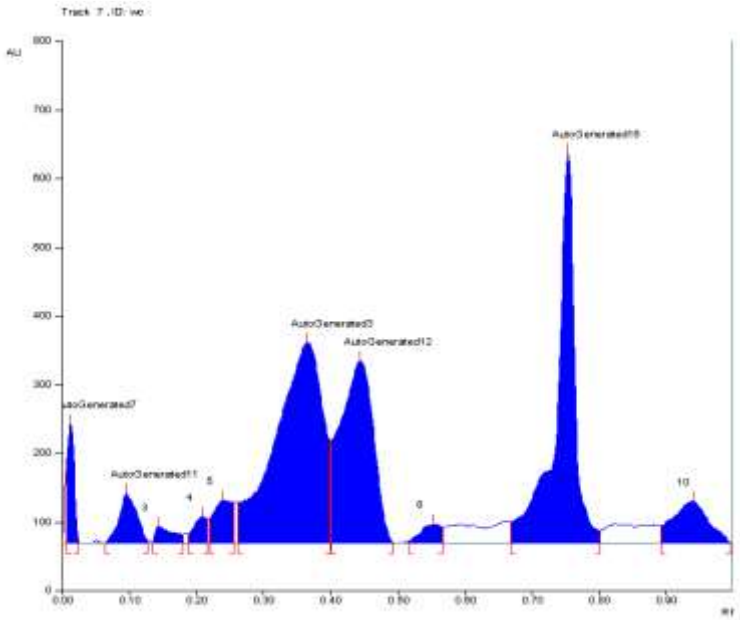
2D Densitograms of each track







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B) Quantitative Analysis: by the Calibration Curve Method

Table.5: Quantitative estimation of Quercetin in HPTLC

Track	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area
1	0.73 Rf	28.1 AU	0.77 Rf	328.7 AU	81.74%	0.79 Rf	19.4 AU	5407.0 AU
2	0.73 Rf	43.0 AU	0.76 Rf	583.0 AU	80.90%	0.79 Rf	23.7 AU	9231.7 AU
3	0.72 Rf	48.3 AU	0.76 Rf	700.5 AU	83.64%	0.79 Rf	27.2 AU	13574.6 AU
4	0.72 Rf	56.1 AU	0.76 Rf	758.5 AU	85.63%	0.81 Rf	15.2 AU	16892.7 AU
5	0.71 Rf	64.6 AU	0.76 Rf	789.5 AU	84.36%	0.80 Rf	19.1 AU	20579.3 AU
6	0.71 Rf	65.1 AU	0.76 Rf	805.5 AU	80.84%	0.81 Rf	19.2 AU	24890.8 AU
7	0.67 Rf	31.4 AU	0.75 Rf	567.7 AU	35.82%	0.80 Rf	17.9 AU	14516.2 AU
8	0.71 Rf	44.6 AU	0.76 Rf	426.1 AU	31.97%	0.79 Rf	61.8 AU	9020.9 AU

Calculation for Quantification:

Table.6 : Results of quantitative estimation in Qurecetin

Concentration (ng/band)	Area
1000	5407.0
2000	9231.7
3000	13574.6
4000	16892.7
5000	20579.3
6000	24890.8

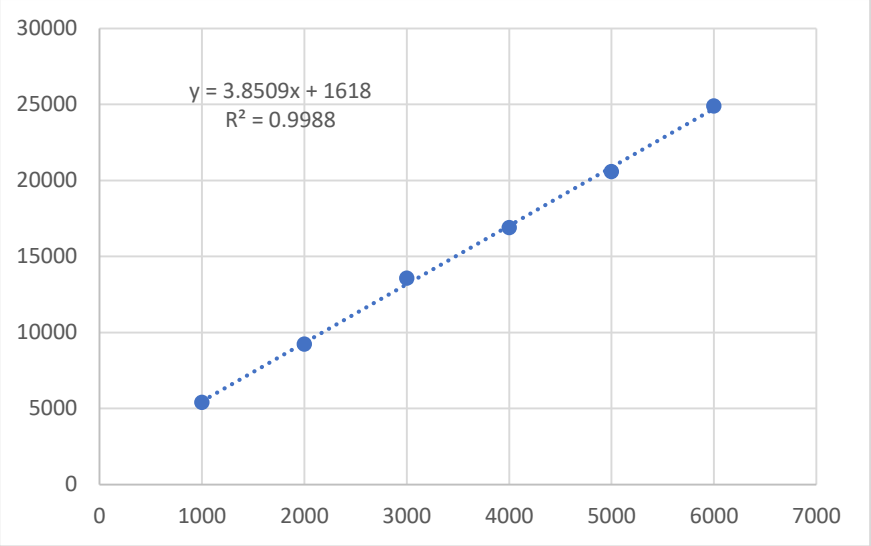

Fig.7: Calibration curve for Quercetin

Table. 7 : Results of Quercetin for plant extracts

				50 µl	1 ml	10 ml	10 ml	10 ml
	Y	m	c	X (ng)	ng	ng	µg	mg
WC	14516.2	3.8509	1618	3349.399	66987.98	669879.768	669.880	0.670
CF	9020.9	3.8509	1618	1922.382	38447.64	384476.356	384.476	0.384

4. DISCUSSION

Studies on the effects of various flavonoids and phenolics in illnesses like pneumonia, cancer, and amoebic dysentery are now being conducted. Quick and accurate assessment of phenolic acids and flavonoids is crucial because of their inherent structural diversity for health and dietary significance.

Total flavonoid content was determined using the aluminium chloride method. Aluminium chloride will form a stable complex with the carbonyl group at C4 and hydroxyls at C3 (flavonols) and C5 in flavonols and flavones. It could also form labile acid complexes with hydroxyls in the ortho position in B rings of flavonoids.

Wedelia chinensis and *Cassytha filiformis* both show significant antioxidant activity due to the presence of polyphenols, phenols, flavonoids, saponins, alkaloids, and glycosides.[4,10]

The present study evaluated the total phenolic contents in two different crude extracts. The most phenolic compounds were present in the *Wedelia chinensis* (155 mg of GAE/g of crude extract), and the lowest was in the *Cassytha filiformis* extract (52.01 mg of GAE/g). *Wedelia chinensis* also had the highest total flavonoid content (72.3 ± 0.233 mgQE/g), while the lowest was in the *Cassytha filiformis* (3.19± 0.256 mgQE/g). In this research, the Rf values of quercetin were found to be 0.75 and 0.76 in *Wedelia chinensis* and *Cassytha filiformis*, respectively, in the HPTLC profile. *This* is equal to the standard biomarker (quercetin), and it shows that the hydroalcoholic extract of *Wedelia chinensis* has more quercetin than the hydralcoholic extract of *Cassytha filiformis*, 0.670 mg and 0.384 mg, respectively. It will help industries and researchers analyse the various herbal formulations.

This suggests that these extracts contain flavonoids and phenolics, which can potentially contribute to managing multiple diseases and their complications. Hence, chemical and *in vitro* pharmacological methods may be considered tools for assisting scientific organisations and manufacturers develop standards.

5. CONCLUSION

Such chemical standards must be established because botanical identification provides limited information for dried or powdered plant material. Hence, considering the analytical methods to find out the authenticity and the reasons behind the therapeutic action of plants. it can be concluded that total phenolics, total flavonoids and HPTLC have proved to be an

effective technique for the herbal drug industry. The present study shows that the rapid confirmation of Quercetin in *Wedelia chinensis* and *Cassytha filiformis* plants by the HPTLC method is possible by using the mobile phase of toluene: ethyl acetate: formic acid (4.3:4.6:1.1 v/v/v).

An authentic, simple, accurate, and specific HPTLC method has been developed to quantitatively estimate quercetin in the whole plant of *Wedelia chinensis* and *Cassytha filiformis*. The data could be used as a Quality Control standard. The method used in this work resulted in good peak shape and resolution of quercetin from other constituents of the plant material.

Conflict of interest: Nil

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