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RESEARCH ARTICLE

# Identification and Quantification of Kaempferol From Ethyl Acetate Fraction of *Pluchea wallichiana* DC Leaves

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## Abstract

*P. wallichiana* DC, commonly known as camphor weed, is practically used in treatment of inflammatory disorders. Identification of kaempferol was done using High-Resolution Liquid Chromatography-Mass Spectrometry (HRLC-MS). High-Performance Thin-Layer Chromatography (HPTLC) method was performed and validated for the quantification of kaempferol. With a correlation coefficient of 0.997, the linearity range for kaempferol was 200 to 700 ng/spot. The suggested method had a recovery rate between 99.60 and 99.85 %. Limit of detection and Limit of quantification were determined to be 25 and 76.47 ng per spot, respectively. The amount of kaempferol was found to be 7.2% in ethyl acetate fraction of leaves. This is the first report of identification and quantification of kaempferol from *P. wallichiana* DC through HRLC-MS and HPTLC methods.

**Keywords:** Ethyl Acetate Fraction, Kaempferol, *P. wallichiana* DC Leaves

## 1. Introduction

As people become more aware of the toxic and negative effects of allopathic drugs so attention in the study as well as the use of medicinal plants has considerably increased globally. Because the utilization of herbs has grown significantly throughout the world, there is a need for scientific confirmation of their pharmacological potential and safety. As a result, research has been done to create plant profiles that show the potency, purity, and safety of herbal remedies[1].

Therefore, it is essential to use scientific standardization techniques for the establishment of the traditional plant profiles.

Natural product standardization involves a number of distinct characteristics that involve both quantitative and qualitative reproducible parameters. Therefore, it is crucial to evaluate these parameters using various physicochemical, chromatographic, spectrophotometric, and biological techniques that can aid in identifying and evaluating the various constituents found in herbal medicines[2]. Phytoconstituents of a medicine that can be used to confirm its identity or potency are referred to as marker compounds. The marker compounds occasionally serve as active chemicals that verify the true botanical identity[3]. Due to the fact that some traditional medicines have active constituents that are unknown and multiple active constituents, it is very challenging to determine the proper marker compounds. Although the concentration of the chemically distinct constituents differ between samples, chromatographic fingerprints can be used to accurately authenticate and identify herbal medicines[4].

Therefore, obtaining consistent chromatographic fingerprints that stand for the active and chemically distinctive components of the herbal drug is crucial[5]. The quality control issue regarding the active phytomarkers can be solved easily using chromatographic fingerprints[6].

The *Pluchea wallichiana* DC species belongs to the genus *Pluchea* that consists of sesquiterpenoids and flavonoids as main constituents. Using column chromatography, Mehdi HK *et al*. reported the presence of β-amyrin, pluviatilol, β-sitosterol, and apigenin as

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important phytoconstituents in this species[7]. So the current study aims to identify and quantify a new marker in *Pluchea wallichiana* leaves. However, HPTLC method for quantification of kaempferol has not been reported in the literature for the same drug.

## 2. Instrument and Chemicals

A system of CAMAG HPTLC with a LINOMAT 5 applicator, 100 µl syringe, a TLC scanner, and Vision CATS users software version 3.0.20196.1 were utilized to determine the constituent. Standard kaempferol (98%) was procured from a Yucca Enterprise (Mumbai), for the development of the method. All the solvents used were of analytical grade.

## 3. Experimental

### 3.1 Collection, Identification and Authentication of Plant

The fresh leaves of the plant were collected from the medicinal plants garden of Parul University in the month of November. Plant authentication was done by Dr Padmanabhi Nagar, M. S. University, Botany Department, Vadodara, Gujarat, India.

### 3.2 Extracts Preparation Using Successive Solvent Extraction

The leaves were subjected to successive solvent extraction. The coarse powder of *Pluchea wallichiana* DC shade dried leaves was extracted using a soxhlet apparatus on a heating mantle for around 48 hours with hexane (HE), chloroform (CHE), ethyl acetate (EAE), and alcohol (AE) in non-polar to polar order until the extraction was completed at a temperature not exceeding 45°C.

### 3.3 Preliminary Detection of Phytoconstituents by Using Thin Layer Chromatography[8]

The thin layer chromatography of the different leaf extracts of the plant was carried out using aluminium silica gel 60 $F_{254}$ sheets as a stationary phase with the use of the reported method to determine the presence of kaempferol as a marker compound. The preliminary testing included TLC of each extract obtained after successive solvent extraction. On the TLC plates, plant extracts (1 mg/ml, each in a volume of 5 µl) and kaempferol as a standard (1 mg/ml, each in a volume of 3 µl) were applied. The solvent front (7 cm) was achieved by using the mobile phase toluene: ethyl acetate: formic acid (12:8:0.6).

### 3.4 Identification of Unknown Phytoconstituents Through HRLC-MS[9-11]

The HR-LCMS analysis of EAE was carried out at Indian Institute of Technology-Sophisticated Analytical Instrument Facility (SAIF) in Powai, Mumbai (India). The HR-MS: Q-TOF-MS B.05.01 version of the instrument was used to conduct the HR-MS analysis of the plant's EAE. The sample was directly injected into the +ESI (Electron spray ionisation) scanner using a syringe pump, and scanned in full scan MS mode to achieve fragmentation. The spectra were recorded for two minutes in positive and negative mode with a voltage of 175.0, which is the ideal voltage for producing ion-source fragments, in the range of 100-950 m/z. Software from the TOF/6500 series was used to identify mass fragments by matching with spectral libraries from the METLIN database.

#### *3.4.1 Statistical Analysis*

The experiments were done in triplicate, and MS Excel 2007 was used to analyze the data as mean ± SD.

### 3.5 Chromatographic Evaluation of EAE[12,13]

The HPTLC quantification of the marker compound in the ethyl acetate extracts of the *P. wallichiana* leaves was done using a syringe (Hamilton) and applicator (LINOMAT 5). Different concentrations of standard (2 -7 µl ) and ethyl acetate extract of leaf as a test solutions (10 and 13 µl) were spotted on a precoated silica gel 60 $F_{254}$ plate as a 5 mm band length. The distance between two bands was 12.4 mm. After 20 minutes of saturation of the solvent system (toluene: ethyl acetate: formic acid - 12: 8: 0.6), the loaded plate was transferred into the TLC twin trough development chamber and allowed to develop in the corresponding mobile phase up to 70 mm after 20 min of saturation. The developed plate was air dried to remove solvents. The images of plate were captured in day light, 254 and 366 nm. Densitometric scanning was performed at 254 nm. The $R_f$ value, the area along with the height of the peak, and densitogram were compared and recorded.

## 3.6 HPTLC Method Development

### 3.6.1 Selection of Solvent

Kaempferol and ethyl acetate extract was dissolved in the methanol and ethyl acetate respectively.

### 3.6.2 Preparation of Standard Stock Solution

The Kaempferol stock solution A (1000 μg/ml) was prepared. From stock solution A, 1 ml was further diluted with 10 ml methanol to get Stock B solution (100 μg/ml) of kaempferol.

### 3.6.3 Preparation of Test Solution

EAE (15 mg) was added in 5 ml ethyl acetate, further subjected to sonication (30 sec) and filtered. 10 μl and 13 μl of the prepared test solution were applied on the TLC plate.

### 3.6.4 Selection of Solvent System

Toluene: ethyl acetate: formic acid (12:08:0.6) was selected depending upon solubility and separation of the kaempferol[12].

## 3.7 Analytical Method Validation[14,15]

The technique was approved for use with a number of parameters in accordance with ICH guideline Q2 (R1).

### 3.7.1 Linearity

Aliquots of 2 to 7 μl of the standard (kaempferol) stock solution B were placed on plates to obtain spots with concentrations of 200 to 700 ng/spot. The graph of concentration v/s peak area were plotted for kaempferol after calculating mean area at each concentration. From the calibration graph regression coefficient equation was obtained.

### 3.7.2 Precision

#### 3.7.2.1 Repeatability

By applying kaempferol (standard) solution (400 ng/ spot) seven times under the same chromatographic conditions, repeatability was verified. Mean of retention time was calculated.

#### 3.7.2.2 Intraday

Three replicates of different concentrations (2, 3, 4, 5, 6 and 7 μl) of kaempferol solution were evaluated at the same day. % RSD was measured with measured peak area.

#### 3.7.2.3 Interday

Three replicates of different concentrations (2, 3, 4, 5, 6 and 7 μl) of kaempferol solution were used to develop chromatogram at the three consecutive days. %RSD was determined with measured peak area.

### 3.7.3 Accuracy

The accuracy was ascertained by calculating the recovery using the standard addition technique. Calculated concentration of test drug (838 ng/ spot, 83.8 μg) and kaempferol solution were spiked at 3 levels (50%, 100% and 150%) with three different concentration of standard (93 ng, 186 ng, and 279 ng/ spot). The amount kaempferol was determined by applying regression equation obtained from the calibration curve. % recovery was calculated from the equation.

### 3.7.4 Robustness

Three replicates of three concentrations (200 ng, 400 ng, and 600 ng) of standard solution of keampferol were analyzed at three different saturation time and three different distance of migration. % RSD was calculated with measured peak area.

### 3.7.5 Limit of Detection (LOD) and Limit of Quantification (LOQ)

Evaluation of LOD and LOQ prove the method's sensitivity and that was estimated with the use of five calibrations curves.

Calculations for the LOD and LOQ include:

LOD = (SD/Slope)*3.3

LOQ = (SD/Slope)*10

Where SD stands for the standard deviation of the five calibration curves' Y-intercepts and mean slope of the five calibration curves is referred to as slope.

# 4. Result

## 4.1 Authentication

The plant of *Pluchea wallichiana* DC was identified and authentified by a plant taxonomist (M. S. University, Vadodara), Dr. Padmanabhi Nagar, where a specimen KMB-1 had been deposited for reference.

## 4.2 Thin Layer Chromatography

The trials with different mobile phases indicate that toluene: ethyl acetate: formic acid (12: 8:0.6) was better than other solvent systems as it is able to give best resolution of the selected standard in the test extract. The result shows that kaempferol is present in EAE (Figure 1).

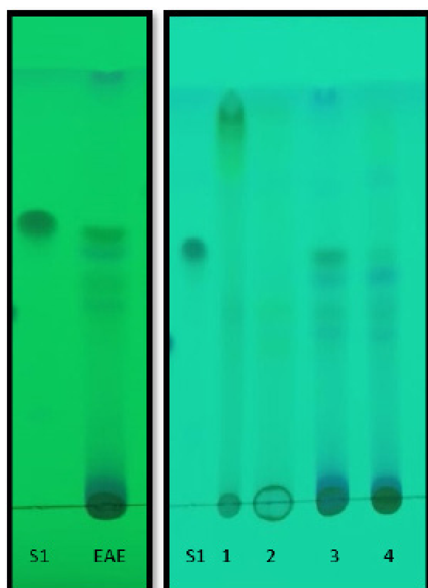


**Figure 1.** TLC profile of, S1-Kaempferol, 1-HE (Hexane Extract), 2-CHE (Chloroform Extract), 3- EAE (Ethyl Acetate Extract) and 4-SAE (alcoholic extract).

## 4.3 Chemical Profiling of Leaf Extract Through HR-LCMS

Further to confirm the presence of phytoconstiuents, HR-LCMS was carried out to identify the chemical constituents present in the EAE of *Pluchea wallichiana.* Compounds were identified based on their molecular formula, molecular mass, retention time and molecular structure by comparing them to known spectra from a spectral library. As a result, compound identification is based on the specific mass of the ion and its match within the available database. The resulting spectrum reveals the presence of several peaks, which are typical of many phytochemicals. HR-LCMS spectra of ethyl acetate extract revealed the major peaks of kaempferol derivatives like compound -28 (Kaempferol 3-O-ß-D galactoside) and compound -48 [Kaempferol 3-(2", 3"-diacetyl-4"-p coumaroyl rhamnoside)] as documented in Figure 2. and 3. The mass spectrum list of compound -28 and compound -48 is mentioned in Tables 1 and 2, respectively, while the retention time of both identified kaempferol derivatives is shown in Table 3.

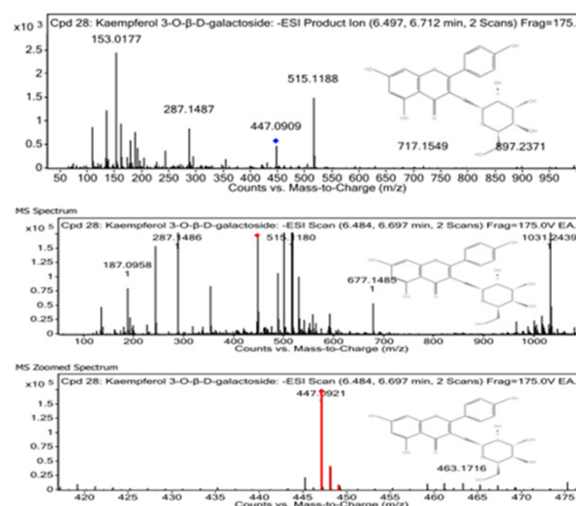


**Figure 2.** HR-LCMS spectrum of compound-28.

**Table 1.** Mass spectrum peak list of compound-28

| Mass spectrum peak list of compound -28- $C_{21} H_{20} O_{11}$ | | | | |
|---|---|---|---|---|
| **M/Z** | **Calculated M/Z** | **Difference (ppm)** | **Formula** | **Ion** |
| 243.1224 | | | | |
| 287.1486 | | | | |
| 447.0921 | 447.0933 | 2.61 | $C_{21} H_{20} O_{11}$ | (M-H)- |
| 448.0952 | 448.0967 | 3.4 | $C_{21} H_{20} O_{11}$ | (M-H)- |
| 449.0984 | 449.0989 | 1.16 | $C_{21} H_{20} O_{11}$ | (M-H)- |
| 499.1232 | | | | |
| 515.118 | | | | |
| 516.1217 | | | | |
| 1031.2439 | | | | |
| 1032.2469 | | | | |

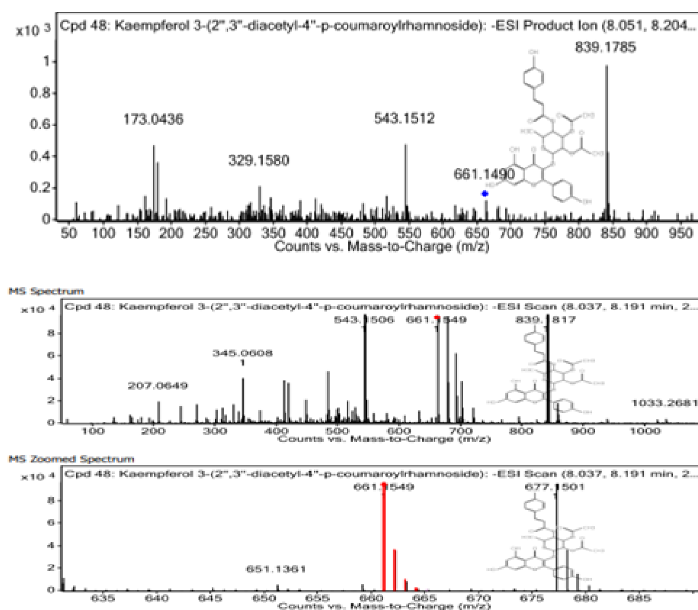


**Figure 3.** HR-LCMS spectrum of compound -48.

**Table 2.** Mass spectrum peak list of compound-48

| **Mass spectrum peak list of compound -48 $C_{34}H_{30}O_{14}$** | | | | |
|---|---|---|---|---|
| M/Z | Calculated M/Z | Difference (ppm) | Formula | Ion |
| 543.1506 | | | | |
| 544.1534 | | | | |
| 661.1549 | 661.1563 | 2.16 | $C_{34}H_{30}O_{14}$ | (M-H)- |
| 662.1578 | 662.1597 | 2.85 | $C_{34}H_{30}O_{14}$ | (M-H)- |
| 663.1619 | 663.1623 | 0.61 | $C_{34}H_{30}O_{14}$ | (M-H)- |
| 664.166 | 664.165 | -1.53 | $C_{34}H_{30}O_{14}$ | (M-H)- |
| 677.1501 | | | | |
| 839.1817 | | | | |
| 840.1855 | | | | |
| 841.188 | | | | |

**Table 3.** Retention time of kaempferol derivatives present in EAE

| **NAME** | **m/z** | **RT** | **Mass** |
|---|---|---|---|
| Kaempferol 3-O-ß-D galactoside | 447.0921 | 6.604 | 448.0994 |
| Kaempferol 3-(2″,3″-diacetyl-4″-p coumaroyl rhamnoside) | 661.1549 | 8.128 | 662.1621 |

## 4.4 HPTLC Analysis and Validation

Quantification of kaempferol in *Pluchea wallichiana* leaves by HPTLC was not reported so far. The solvent system of toluene, ethyl acetate, and formic acid (12: 8: 0.6) provided better, sharper, and more defined resolution. The spot at $R_f$ (0.62) was recognized as kaempferol using standard compound's $R_f$ (0.61) after 20 minutes chamber saturation at ambient temperature (Figure 4). The TLC plate was visualized at 254 nm. TLC plate with standard (kaempferol) and EAE separation was photographed. Figure 5 shows a 3D chromatogram of EAE and kaempferol standards for further verification.

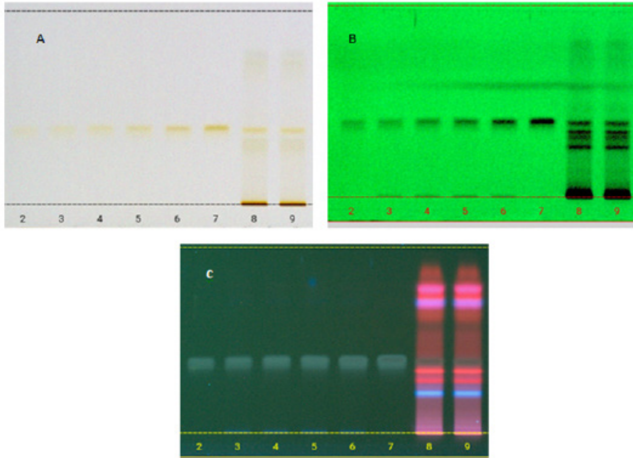


**Figure 4.** Fingerprinting of EAE and kaempferol [(A) White light (B) 254 nm (C) 366 nm ; track 2-7 Kaempferol (standard); track 8,9 EAE].

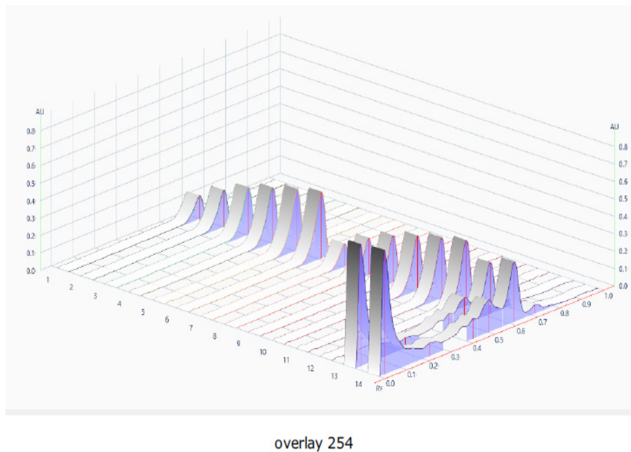


**Figure 5.** 3D Densitometric chromatogram of ethyl acetate extract and kaempferol.

## 5. Validation of Method

### 5.1 Linearity

The various concentration series (200-700 ng/band) of standard was utilized to determine the linearity. The calibration curve of concentration versus area was plotted that was observed to be linear, with a 0.997 correlation coefficient (Figure 6, Table 4).

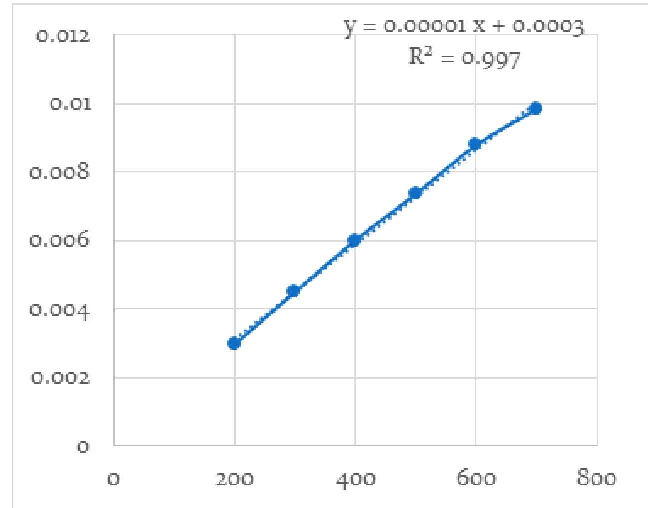


**Figure 6.** Calibration curve of kaempferol.

**Table 4.** Linearity

| Replicates | Concentration of Kaempferol (ng) | | | | | |
|---|---|---|---|---|---|---|
| | **200** | **300** | **400** | **500** | **600** | **700** |
| 1 | 0.0029 | 0.00457 | 0.00617 | 0.00732 | 0.00892 | 0.0098 |
| 2 | 0.00297 | 0.0045 | 0.00601 | 0.00736 | 0.00879 | 0.00984 |
| 3 | 0.00293 | 0.00472 | 0.00617 | 0.00766 | 0.00899 | 0.00969 |
| 4 | 0.0029 | 0.00461 | 0.00601 | 0.00735 | 0.00883 | 0.00976 |
| 5 | 0.00297 | 0.00454 | 0.00602 | 0.00736 | 0.0088 | 0.00979 |
| **Mean** | 0.002934 | 0.004588 | 0.006076 | 0.00741 | 0.008866 | 0.00984 |
| **SD** | 0.000035 | 0.000084 | 0.000086 | 0.000141 | 0.000086 | 0.000056 |
| **%RSD** | 1.195343 | 1.83267855 | 1.413873048 | 1.898954 | 0.972225 | 0.5685 |

## 5.2 Repeatability

Repeatability was studied by applying seven band of kaempferol (400 ng/spot) with the RSD < 2% (Table 5).

**Table 5.** Repeatability

| Concentration (ng/spot) | Area |
|---|---|
| 400 | 0.0131 |
| 400 | 0.01294 |
| 400 | 0.01282 |
| 400 | 0.0131 |
| 400 | 0.01269 |
| 400 | 0.013 |
| 400 | 0.01267 |
| **Mean** | 0.012903 |
| **SD** | 0.00018 |
| **%RSD** | 1.396986 |

## 5.3 Precision

The mean % RSD value was 0.79% and 1.29% for intra and inter day precision respectively. RSD values were under 2% in every case, supporting the accuracy method (Table 6).

**Table 6.** Precision

| | Intra day | | | Inter day | | |
|---|---|---|---|---|---|---|
| Conc. (µl) | Area | Avg±SD | %RSD | Area | Avg±SD | %RSD |
| 200 | 0.00285 | 0.00285±0.00002 | 0.535348 | 0.00312 | 0.002853±1.3 | 1.3268 |
| | 0.00287 | | | 0.00319 | | |
| | 0.00284 | | | 0.00313 | | |
| 300 | 0.00487 | 0.0048±0.000042 | 0.860785 | 0.00472 | 0.00472±0.63 | 0.6355 |
| | 0.00485 | | | 0.00469 | | |
| | 0.00479 | | | 0.00475 | | |
| 400 | 0.00647 | 0.00642±0.000042 | 0.648158 | 0.00617 | 0.006207±1.16 | 1.1655 |
| | 0.00641 | | | 0.00629 | | |
| | 0.00639 | | | 0.00616 | | |
| 500 | 0.00792 | 0.00791±0.00005 | 0.636043 | 0.00766 | 0.007667±1.43 | 1.4367 |
| | 0.00796 | | | 0.00778 | | |
| | 0.00786 | | | 0.00756 | | |
| 600 | 0.00932 | 0.00923±0.000119 | 1.29163 | 0.00899 | 0.008853±1.71 | 1.7142 |
| | 0.00929 | | | 0.00869 | | |
| | 0.0091 | | | 0.00888 | | |
| 700 | 0.01068 | 0.01072±0.000102 | 0.952537 | 0.01122 | 0.011177±1.51 | 1.5139 |
| | 0.01084 | | | 0.01132 | | |
| | 0.01065 | | | 0.01099 | | |

## 5.4 Robustness

The analysis was performed under the change in condition like chamber saturation time and distance of migration and did not show any major deviation. (% RSD < 2) (Table 7).

**Table 7.** Robustness

| Drug | Chamber saturation time (min.) | | | Distance of migration (mm) | | |
|---|---|---|---|---|---|---|
| Kaempferol | 22 | 23 | 24 | 74 | 76 | 78 |
| | 0.01262 | 0.0137 | 0.01294 | 0.0137 | 0.01317 | 0.01367 |
| | 0.01238 | 0.01348 | 0.01282 | 0.01338 | 0.01318 | 0.0132 |
| | 0.01222 | 0.01345 | 0.01269 | 0.01341 | 0.01335 | 0.01355 |
| **Mean** | 0.012407 | 0.01354333 | 0.012816667 | 0.013497 | 0.013233 | 0.013473333 |
| **S.D.** | 0.000201 | 0.0001365 | 0.000125033 | 0.000177 | 0.000101 | 0.000244 |
| **%RSD** | 1.622748 | 1.00790525 | 0.975552631 | 1.309431 | 0.764433 | 1.812464414 |

## 5.5 Accuracy

The accuracy was calculated using the recovered and predicted concentrations. The recovery of the suggested method was good, as evidenced by the average recovery being in the desirable range from 99.60 and 99.85 % (Table 8).

**Table 8.** Accuracy

| Amount | Standard (ng) | Test (ng) | Area | Avg ±SD | Recovered | % Recovery | % Average recovery |
|---|---|---|---|---|---|---|---|
| **50%** | 93 | 279 | 0.003082 | 0.003096±0.001 | 278.2 | 99.713 | 99.60 |
| | 93 | 279 | 0.003105 | | 280.5 | 100.537 | |
| | 93 | 279 | 0.0031 | | 275 | 98.566 | |
| **100%** | 186 | 372 | 0.004011 | 0.004023±0.0012 | 371.1 | 99.758 | 99.856 |
| | 186 | 372 | 0.004038 | | 370.8 | 99.677 | |
| | 186 | 372 | 0.00402 | | 372.5 | 100.134 | |
| **150%** | 279 | 465 | 0.007664 | 0.005832±0.0015 | 465 | 100 | 99.79 |
| | 279 | 465 | 0.004971 | | 463.1 | 99.591 | |
| | 279 | 465 | 0.00486 | | 464 | 99.7849 | |

## 5.6 LOD and LOQ

The LOD and LOQ of kaempferol to be 25 and 76.47 ng/spot, respectively (Table 9).

**Table 9.** LOD and LOQ study

| Parameters | |
|---|---|
| **Standard deviation of Y intercepts** | 0.00013 |
| **Mean slope of calibration curves** | 0.000017 |
| **LOD = 3.3 (SD/slope) (ng/spot)** | 25.23529 |
| **LOQ = 10 (SD/slope) (ng/spot)** | 76.47059 |

## 5.7 Estimation of Kaempferol in EAE

HPTLC result revealed that 7.02 ± 0.002 % w/w kaempferol was present in ethyl acetate extract with the average peak area of 0.01080 ± 0.00013 (Table 10).

**Table 10.** Quantitative estimation of kaempferol in EAE

| Extract | Average peak area | Amount (µg/ spot) | Concentration % w/w |
|---|---|---|---|
| **Kaempferol** | | | |
| **Ethyl acetate** | 0.01080 ± 0.00013 | 1.05 ± 0.0134 | 7.02 ±0.002 |

## 6. Discussion

Assessment of HPTLC chromatogram patterns appears promising for locating active compounds in plant extracts. HPTLC is a tool that assures the quality and presence of active compounds in plant extracts. Additionally, HPTLC is used to obtain a unique chromatographic fingerprint for the identification and quantification of plant extract actives under ideal experimental conditions.

An HPTLC method was developed to measure the amount of kaempferol in *Pluchea wallichiana* DC leaves and provides dense, compact spots with significant $R_f$ values. According to polarity order, various solvents, such as HE, CHE, EAE, and AE, were used in the current study. Because flavonoids are polar compounds, polar solvents, such as ethyl acetate was used to extract them more effectively than non-polar ones. TLC results suggest the presence of kaempferol in the EAE out of all the tested extracts. The presence of kaempferol in the ethyl acetate fraction was justified further by HR-LCMS. For the separation and quantification of kaempferol from *Pluchea wallichiana* DC, however, not a single report has been cited. To support the aforementioned claim, an HPTLC method for the standard marker kaempferol in the EAE of *Pluchea wallichiana* DC leaves was investigated. According to the results of the HPTLC analysis, kaempferol was identified because $R_f$ value (0.61) of the standard was closely related with the $R_f$ (0.62) value of kaempferol in EAE. The percentage of kaempferol was revealed to be 7.2% in the ethyl acetate fraction. The ability of an analytical method to produce test results that are directly proportional to the analyte concentration in samples falling within a given range, or that do so after undergoing a clearly defined mathematical transformation, is referred to as linearity. By analyzing standard (kaempferol) solutions ($1\,mg\,ml^{-1}$) at six different concentration levels, ranging from 200 to 700 ng/spot, the linearity was evaluated with a correlation coefficient of 0.997. The LOQ establishes the least concentration at which the analyte can be accurately measured, the LOD was definite based on the minimum concentration detected by the instrument from standard, but not necessarily quantified. LOQ and LOD were discovered to be 25.24 and 76.47 ng/spot. The intra-day and the inter-day RSD were 0.79%, and 1.29%, respectively. The percentage of recovery from the assay of the recognized additional quantity of standard in the test sample was used to calculate accuracy. The method was considered accurate because, in EAE, the average per cent recovery was 99.7%. Instrumental precision was checked by analyzing spots derived from applying seven 400 ng of standard kaempferol solution. The robustness is an assessment of its ability to be unchanged by little but intentional variations in parameters and offers a clue as to how reliable it will be under typical conditions. The method was found to be robust as the % RSD was less than two. The HPTLC method for kaempferol analysis in *Pluchea wallichiana* DC. leaves were reported for the first time. The parameters and information provided by this HPTLC method are sufficient to identify and quantify kaempferol completely. However, it is necessary to continue these studies using some other common markers in the EAE to investigate phytoactive that may have therapeutic prospects in a number of illnesses.

## 7. Conclusion

In the present study, kaempferol was identified as a marker compound in the ethylacetate fraction of *Pluchea wallichiana* leaves for the first time through HR-LCMS and HPTLC. It is important to confirm the quantity due to the variety of environmental factors and the nature of phytoactive. So the quantitative determination of flavonoid (kaempferol) in *Pluchea wallichiana* DC was done by HPTLC.

## 8. Acknowledgement

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## 9. References

1. Saggar S, Mir PA, Kumar N, Chawla A, Uppal J, Kaur A. Traditional and herbal medicines: opportunities and challenges. Pharmacogn Res. 2022; 14(2):107-14. https://doi.org/10.5530/pres.14.2.15
2. Kumari R, Kotecha M. A review on the standardization of herbal medicines. International Journal of Pharma Sciences and Research. 2016; 7(2):97-106.
3. Chothani DL, Patel MB, Mishra SH. HPTLC-fingerprint profile and isolation of marker compound of *Ruellia tuberosa*. Chrom Res Int. 2012; 2012:1-6. https://doi.org/10.1155/2012/180103

4. Patil PS, Shettigar R. An advancement of analytical techniques in herbal research. Journal of Advanced Scientific Research. 2010; 1(01):8-14.
5. Liang Y-Z, Xie P, Chan K. Quality control of herbal medicines. Journal of chromatography B. 2004; 812(1-2):53-70. https://doi.org/10.1016/S1570-0232(04)00676-2
6. Ong ES. Chemical assay of glycyrrhizin in medicinal plants by Pressurized Liquid Extraction (PLE) with Capillary Zone Electrophoresis (CZE). Journal of Separation Science. 2002; 25(13):825-31. https://doi.org/10.1002/1615-9314(20020901)25:13%3C825::AID-JSSC825%3E3.0.CO;2-I
7. Kazmi MH, Khan S, Hameed S. Antimicrobial constituents from *Pluchea wallichiana* DC. Int J Bioand Biotechnol (Pakistan). 2012; 9(3):213-8
8. Mali PY, Goyal S. HPTLC densitometric quantification of kaempferol from leaves of euphorbia neriifolia. Indian J Pharm Educ Res. 2020; 54:s586-s92. https://doi.org/10.5530/ijper.54.3s.158
9. Neupane P, Lamichhane J. Phytochemical profiling using HRLCMS and evaluation of antioxidant and antibacterial activities of Nepalese medicinal plants. Vegetos. 2020; 33(4):628-40. https://doi.org/10.1007/s42535-020-00143-4
10. Lucci P, Saurina J, Núñez O. Trends in LC-MS and LC-HRMS analysis and characterization of polyphenols in food. TrAC Trends in Analytical Chemistry. 2017; 88:1-24. https://doi.org/10.1016/j.trac.2016.12.006
11. Shah MB. High Resolution-Mass Spectrometry (HR-MS) analysis of *Bryonia laciniosa* . Indian Journal of Natural Products and Resources (IJNPR) [Formerly Natural Product Radiance (NPR)]. 2022; 12(4):617-24.
12. Panchal H, Amin A, Shah M. Development of validated high-performance thin-layer chromatography method for simultaneous determination of quercetin and kaempferol in Thespesia populnea. Pharmacognosy Research. 2017; 9(3):277. https://doi.org/10.4103/0974-8490.210326 PMid:28827970 PMCid:PMC5541485.
13. Bhandare SB, Laddha KS. Simultaneous quantification of kaempferol and quercetin in medicinal plants using HPTLC. International Journal of Pharmaceutical Sciences and Research. 2016; 7(6):2379.
14. Laila O, Murtaza I, Abdin M, Ahmad S, Ganai NA, Jehangir M. Development and validation of HPTLC method for simultaneous estimation of diosgenin and quercetin in fenugreek seeds (*Trigonella foenum-graceum*). International Scholarly Research Notices. 2014;2014. https://doi.org/10.1155/2014/583047
15. Guideline ICH validation of analytical procedures Q2 (R1). International Conference of Harmonization Geneva, Switzerland; 2022.