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# DEVELOPMENT AND VALIDATION OF RP-HPLC-PDA METHOD FOR IDENTIFICATION AND QUANTIFICATION OF MAJOR ACTIVE CONSTITUENT IN *CYNODON DACTYLON* (L.) PERS.

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**ABSTRACT**. In this research, two primary bioactive components were isolated from *Cynodon dactylon* (L) Pers, commonly known as *Durva* grass. A gradient reverse phase High performance liquid chromatography coupled to Photodiode array detector (RP-HPLC-PDA) technique was established to identify and quantify these major bioactive constituents in 70% hydroalcoholic extracts of *Cynodon dactylon* (L) Pers. The chromatographic separation was achieved using an RP-C18 column (250 mm × 4.6 mm, 5 µm), Alliance (Waters) Empower3 liquid chromatography system, and a gradient mobile phase consisting of 5% acetic acid and acetonitrile. The flow rate was set at 1.2 mL/min, and a PDA detector at 320 nm was employed to analyze column effluents, targeting the active compounds' isotactic point. The retention times for the compounds *p*-coumeric and ferulic acid were determined as 24.11 and 31.52 min. The method exhibited linearity within the concentration range of 2–10 µg/mL, with regression coefficients of 0.998 and 0.9967 for the respective compounds. The mean recovery of *p*-coumeric and ferulic acid were found to between 102.64 and 109.44%. The RP-HPLC method was validated in accordance with ICH guidelines.

**KEY WORDS**: 4-Hydroxycinnamic acid, Durva grass, Bioactive, Liquid-liquid extraction, RP-HPLC

## INTRODUCTION

In recent years, there has been a renewed global interest in exploring non-traditional approaches for treating diseases. Extensive evidence gathered thus far highlights the substantial potential of medicinal plants utilized in traditional systems. *Cynodon dactylon*, commonly known as *Durva* grass, holds significant medicinal value and can be administered both externally and internally. The plant has demonstrated antiviral, anti-diabetic, and antimicrobial activities [1]. Traditionally, decoctions of its roots were employed for treating secondary syphilis and irritation of urinary organs.

*Cynodon dactylon* exhibits a range of properties, being astringent, sweet, cooling, haemostatic, depurative, vulnerary, constipating, diuretic, and tonic. It has proven beneficial in conditions associated with impaired pitta and kapha, addressing issues such as hyperdipsia, burning sensation, haemoptysis, haematuria, haemorrhages, wounds, leprosy, diarrhea, dysentery, conjunctivitis, vomiting, snake bites, gout, and rheumatic affections [2-3]. The plant's anthelmintic activity has been thoroughly investigated and validated [4]. Furthermore, *Cynodon dactylon* demonstrates anti-inflammatory properties.

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The plant is classified into three varieties: 'nildurva,' characterized by a bluish or greenish stem; 'shvetadurva,' distinguished by a whitish stem and branches; and 'gandadurva,' featuring a nodulose stem, as mentioned in the 'Bhavaprakash Nighantu.' *Cynodon dactylon* thrives in warm climates globally, spanning from 45° south to north latitudes, and is available throughout the year.

Various methods have been suggested only for the quantitative estimation of *p*-coumeric acid in *Cynodon dactylon* (L) Pers. [5]. The estimation of most therapeutic bioactive compounds of *p*-coumeric and ferulic acid were developed and validated by HPLC (high-performance liquid chromatographic) in *Cynodon dactylon* (L) Pers. The RP-HPLC technique has several advantages over other methods such as simple, non-destructive, sample recovery and rapid separation [6, 7]. The methods described above have several limitations like tedious sample preparation. In the present study, accurate, simple, and reproducible HPLC method has been developed and validated for the determination of *p*-coumeric and ferulic acid in *Cynodon dactylon* (L) Pers. herb and extracts [8, 9].

## EXPERIMENTAL

### *Materials*

Standards *p*-coumeric and ferulic acid were procured from Sigma (purity 98 and 99 %). Methanol, acetonitrile were HPLC grade, analytical grade acetic acid were purchased from sd-fine chemicals (Mumbai). All the solutions used for HPLC analysis were filtered through 0.45 µm membrane syringe filter. Whole plant of *Cynodon dactylon* (L) Pers. was authenticated by botanist, The Himalaya Drug Company, and Bangalore.

### *Instrumental techniques*

UV spectrophotometer and GC/MS were Shimadzu instruments, HPTLC were CAMAG, Purified water generated by the ELGA System Model LA611 was used. 5% Acetic acid (solvent A) and acetonitrile (solution-B) used as mobile phase for HPLC analysis.

### *Preparation of plant extract*

The entire *Cynodon dactylon* plant was utilized in this study. To ensure the purity of the material, it underwent a thorough cleaning process to eliminate molds, insects, animal fecal matter, and other potential contaminants such as earth, stones, and extraneous materials. Subsequently, the plant material was shade-dried, protected from sunlight for over one month, and then finely powdered using a pulverizer. The resulting powder was sieved through a 40-mesh sieve, and the particles that passed through the sieve were collected and stored in a well-sealed amber-colored container for further analysis.

Approximately 2 kg of the 40-mesh powder was subjected to successive extraction using a continuous Soxhlet apparatus with 70% ethanol at 80 °C for 24 h. The resulting homogenous solution was filtered using Whatman's filter 45 paper, and the solvent was concentrated and dried at 80 °C on an evaporation water bath. The dried extract was then stored in vacuum desiccators for future use. The obtained dry extract ratio (DER) from the raw material was approximately 10-12%, as reported in references [10-12]. These extracts were subsequently employed in various aspects of the study.

### *Calibration curve of standard (p-coumeric and ferulic acid) and sample*

In this procedure, 10 mg of both *p*-coumaric and ferulic acid standards were individually weighed and placed in 10 mL volumetric flasks. These standards were dissolved by sonication

for 2 min with 7 mL of methanol, and the volume was adjusted with methanol to create stock solutions with a concentration of 1 mg/mL. Working standard solutions were then prepared by diluting the stock solutions with methanol within the concentration range of 20–100 µg/mL (0.02–0.1 mg/mL). Triplicates of 20 µL from each working standard solution were injected, and a calibration curve was constructed through linear regression based on the observed peak areas. For the extract solution, concentrations ranging from 2.5 mg/mL to 7.5 mg/mL were prepared in diluent, and 20 µL from each solution was injected in triplicates.

*HPLC instrumentation*

The HPLC system used in this study was Alliance Waters quaternary pumps, a PDA, auto-injector, and a C18 ODS ACE-Generix column (250 x 4.60 mm, 5µm) maintained at a temperature of 35 °C. The chromatographic separation was achieved through gradient elution with a mobile phase consisting of 5% acetic acid in water (A) and acetonitrile (B). The solvent composition varied over time: from 0.0 to 3.0 min, it ranged from 0% to 8.5%; 3.0 to 8.0 min saw a decrease to 2%; 8.0 to 21.0 min increased to 5%; 21.0 to 33.0 min rose to 15%; 33.0 to 40.0 min declined to 0%; and 40.0 to 45.0 min maintained at 0%. The flow rate was set at 1.2 mL/min, and detection was performed at 320 nm using a PDA. Empower 3 software facilitated integration and validation, with the assessment based on peak areas using linear regression.

*Estimation of p-coumeric and ferulic acid in fractionated hydroalcoholic extract*

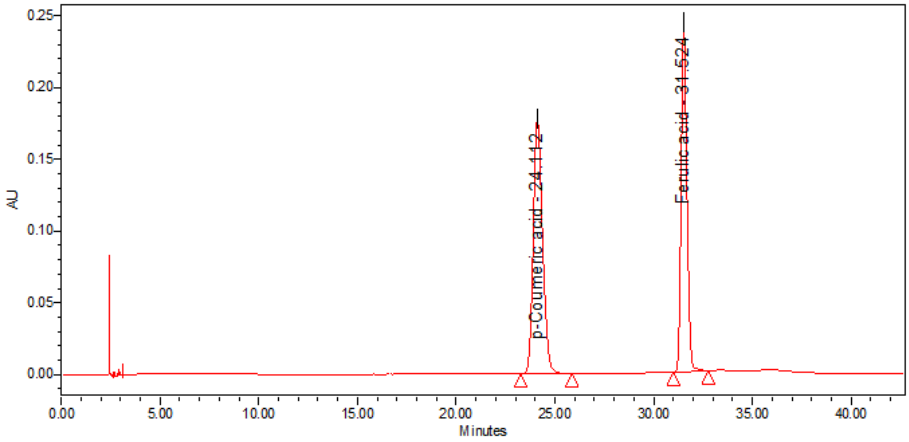
About 10 g of hydroalcoholic extract was weighed accurately in a 100 mL beaker. About 50 mL of water was added and sonicated for about 10 min. The solution was adjusted pH 2 using concentrated hydrochloric acid and transferred into a 250 mL separating flask. The aqueous solution was extracted thrice in the combination of ethyl acetate : petroleum ether : hexane (80:40:40; 40:80:40; 40:40:80), the solvents were collected through Whatman No. 1 filter paper in 1000 mL beaker, finally the aqueous solution were fractionated with chloroform 100 x 3 times. The solvents were combined in same 1000 mL beaker and evaporated up to dryness on evaporation water bath at 80 °C [13-16]. Exactly, 50 mg of dried extract weighed into 10 mL volumetric flask and 7 mL of methanol added and sonicated for 5 min. The volume was made up with same solvent and filtered through 0.45 µ membrane filter. First 2 mL of filtrate discarded and the subsequent solution used for the analysis. An aliquot of 20 µL of sample were injected in to HPLC. The HPLC analysis was continued for 45 min run time. The content of *p*-coumeric and ferulic acid was calculated by linear regression and mean percentages were calculated from triplicate experiments. The percentage of *p*-coumeric and ferulic acid calculated by using 0.05 and 0.005 mg/mL of *p*-coumeric and ferulic acid mixed standard. The chromatograms are shown in Figure 1A and B. The PDA spectrums are shown in Figure 2.

*Identification of p-coumeric and ferulic acid by high performance thin layer chromatography (HPTLC) method*

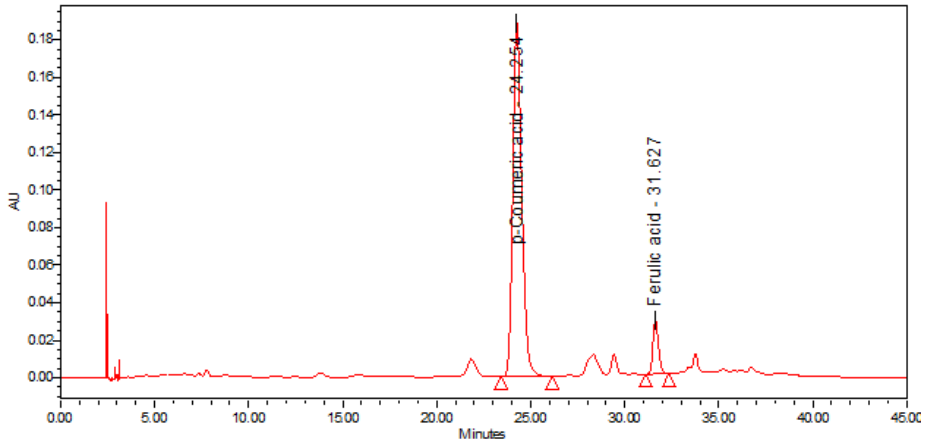
About 0.1 g of plant powder and 50 mg of extract were taken in separate 10 mL volumetric flask and sonicated 10 min with 8 mL of methanol and made up to 10 mL with same solvent. Reference standard of *p*-coumeric and ferulic acid prepared at concentration of 0.1 mg/mL in methanol. An aliquot of 10 µL of sample and standard were spotted on CAMAG HPTLC Linomat V as 12 mm band width on a precoated Silica gel 60 $F_{254}$ plate of thickness (0.2 mm). The plate was developed in the solvent system chloroform: methanol: formic acid (85:15:1) in the pre-saturated chromatographic chamber. The developed plate were dried under normal air and the spots were visualized at 254 and 366 nm UV light. Identification of the band of *p*-coumeric and ferulic acid was captured and the $R_f$ values of raw material, extract and standards were calculated. [17-19].

*Preparative TLC for purification from fractionated hydro alcoholic extract*

An automated system deposited an extract streak onto a preparative TLC glass plate (20 cm × 20 cm; 1500 film thickness). Following air drying, the plate underwent development in a pre-saturated glass chamber using the same mobile phase employed in the analytical HPTLC. In each iteration, two plates ran in parallel, and the bands were carefully scraped off from the plate. The scraped samples were then dissolved in HPLC-grade methanol and subjected to centrifugation at 4000 rpm for 20 min to eliminate silica. The resulting supernatant was collected, filtered using a 0.45 syringe filter, and dried under reduced pressure. Subsequently, the dried samples were dissolved in methanol for further characterization and qualitative HPLC analysis. The chromatograms depicting the isolated compounds of *p*-coumaric and ferulic acid from the extract are presented in Figure 3.

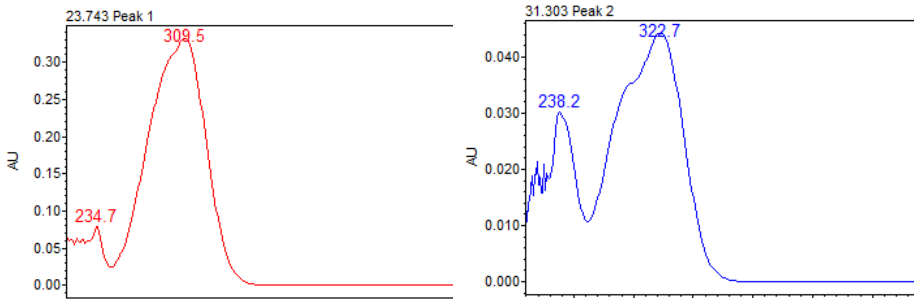



A) Standard *p*-coumeric and ferulic acid




B) Extract sample *p*-coumeric and ferulic acid

Figure 1: A) *p*-Coumeric and B) ferulic acid from fractionated hydroalcoholic extract.




*p*-Coumeric acid Ferulic acid

Figure 2. PDA spectrum of compounds extracted.

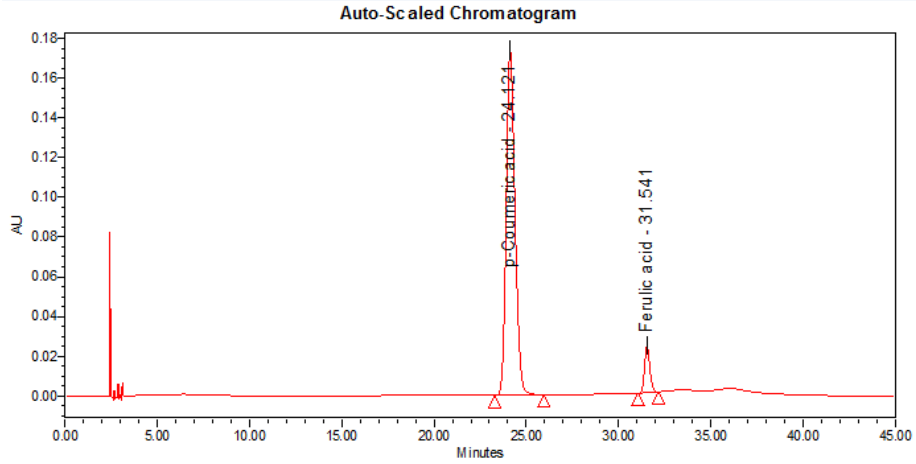



Figure 3. Isolated compound of *p*-coumeric and ferulic acid.

*Characterization of p-coumeric and ferulic acid in sample*

The UV spectrum of the compounds were recorded between 200 and 600 nm on double beam UV-Visible spectrophotometer for the above centrifuged supernatant liquid. The results are mentioned in Table 1.

The m/z spectra values of *p*-coumaric acid and ferulic acid in the sample were determined using a Shimadzu Model 7890A gas chromatograph, equipped with a split/splitless injection port. A fused silica (DB-5MS) capillary column with dimensions 30 m × 0.25 mm i.d., 0.25 mm was utilized. Helium served as the carrier gas, with a flow rate set at 1.2 mL/min and a column head pressure of 26.00 psi. The injector temperature was maintained at 320 ºC, and the sample injection volume was 2 µL with a 1:50 split ratio. The interface temperature was held at 280 ºC. The column temperature program involved an equilibration time of 2 min, an initial temperature of 150 ºC for 3 min, an increase to 292 ºC at a rate of 5 ºC/min, and then to 320 ºC at a rate of 30 ºC/min with a final isotherm of 4 min. GC-MS analysis of the extracts was conducted in scan mode. Peak identification was achieved by comparing the retention times with those of

commercial standards (*p*-coumaric, ferulic acid) and the spectral data provided in Figure 4. The obtained results are presented in Table 2 [20].

Table 1. Lambda max spectrum value of purified compounds by preparative TLC.

| Compound | Scanning range | Lambda max (nm) |
|---|---|---|
| *p*-Coumeric acid | 200 - 600 | 309 |
| Ferulic acid | 200 - 600 | 323 |

Table 2. m/z value of purified compounds by preparative TLC.

| Compound | Molecular weight (MW) | Molecular ion $M^+$ | Fragmented ions (m/z) |
|---|---|---|---|
| *p*-Coumeric acid | 164.2 | 164.2 | 242 (100), 178 (43), 120 (12.2), 40 (19) |
| Ferulic acid | 191 | 191 | 306 (100), 198 (43), 163 (12.2), 60 (19) |

## RESULTS AND DISCUSSION

Due to the intricate chemical composition of herbal extracts, ensuring the quality of these extracts necessitates the implementation of validated analytical methods for the identification and quantification of active ingredients. In this context, the HPLC methods employed for the quantitative assessment of *p*-coumaric and ferulic acid underwent validation procedures to assess their precision, accuracy, and linearity.

### *HPLC method validation p-coumeric and ferulic acid*

The development of a suitable mobile phase is a crucial step in formulating an analytical procedure. In this study, the composition of the HPLC mobile phase was optimized to achieve optimal resolution. Several runs with varying % of acetic acid were employed to obtain a satisfactory peak shape and check the robustness of the mobile phase in achieving the desired goals. The most favourable resolution and peak shape were attained using a mobile phase comprising 5% acetic acid in water (solvent-A) and acetonitrile (solvent-B). The compounds with a specific retention time were identified at 24.1 and 31.5 min corresponding to *p*-coumeric and ferulic acid (Figure 1A). The additional spikes observed at various retention times noticed in Figure 1B are dues to other biomolecules of the extract. Precision, reflecting the reproducibility or repeatability of the analytical method, was evaluated through intra- and inter-day variations. Triplicates of three concentrations of the extract were analyzed on the same day (intra-day) and on different days (inter-day), expressed as percent relative standard deviation (% RSD). The results revealed insignificant intra- and inter-day variations, with % RSD values ranging from 0.11% to 0.90% and 0.1% to 0.4%, respectively, all falling below 2%. Detailed results are presented in Table 3.

Accuracy was assessed through recovery studies, wherein pre-analyzed samples were spiked with three different concentrations of standard *p*-coumaric and ferulic acid (10%, 20%, and 30%). The mixtures were then analyzed by the proposed method, yielding recoveries within the range of *p*-coumaric acid 106.95 - 108.70%, 107.68 - 109.53%, and 100.83 - 102.19%, and ferulic acid 106.95 - 108.70%, 107.68 - 109.53%, and 100.83 - 102.18%. The average recovery percentages were *p*-coumaric acid 107.72 ± 0.80%, 108.44 ± 0.78 %, and 101.64 ± 0.769%, and ferulic acid 106.72 ± 0.79%, 108.44 ± 0.88%, and 102.64 ± 0.69%. Detailed results are provided in Table 4.

The linearity of the standard curves was assessed by injecting five standard working solutions (20 - 100 µg/mL) and five concentrations of the sample (50-150%, i.e., 2.5 mg/mL to 7.5 mg/mL solutions). Least square linear regression analysis was applied to calculate the calibration equation and correlation coefficient. Linearity was achieved over a concentration range of 20 - 100 µg/mL for standards and 50 - 150% for the sample. The sensitivity of the method was evaluated by estimating the limit of detection (LOD) and limit of quantification (LOQ) based on signal-to-noise ratios of 3:3 and 10, respectively. Results are outlined in Table 5. The observed theoretical plates, resolution, and asymmetry met the criteria outlined in the USP (2006) system suitability standards page No: 2647 [21].

Table 3. Intra and inter-day precision of *p*-coumeric and ferulic acid.

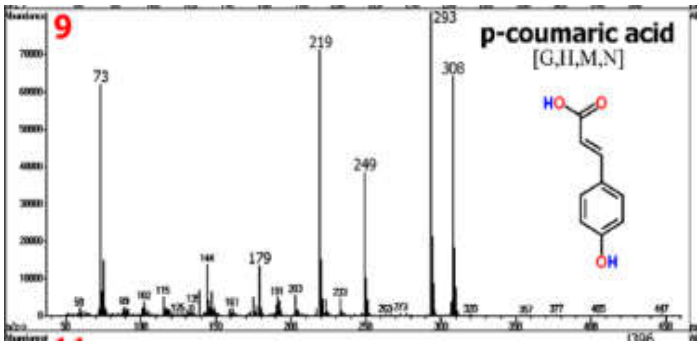
| Analysis on day-1 (%) | | | | Analysis on day-2 (%) | | | |
|---|---|---|---|---|---|---|---|
| Repeatability sample | p-Coumeric acid | Ferulic acid | Mean ± %RSD | Intermediate precision | p-Coumeric acid | Ferulic acid | Mean ± %RSD |
| 1 mg/mL | 1.078 | 0.123 | - | 1 mg/mL | 1.078 | 0.123 | - |
| 1 mg/mL | 1.066 | 0.129 | - | 1 mg/mL | 1.066 | 0.129 | - |
| 1 mg/mL | 1.082 | 0.122 | 1.07 ± 0.18, 0.12 ± 0.14 | 1 mg/mL | 1.082 | 0.122 | 1.07 ± 0.18, 0.12 ± 0.14 |
| 3 mg/mL | 1.065 | 0.124 | - | 3 mg/mL | 1.065 | 0.124 | - |
| 3 mg/mL | 1.073 | 0.122 | - | 3 mg/mL | 1.073 | 0.122 | - |
| 3 mg/mL | 1.069 | 0.122 | 1.06 ± 0.12, 0.12 ± 0.09 | 3 mg/mL | 1.069 | 0.122 | 1.06 ± 0.12, 0.12 ± 0.09 |
| 5 mg/mL | 1.088 | 0.123 | - | 5 mg/mL | 1.088 | 0.123 | - |
| 5 mg/mL | 1.079 | 0.128 | - | 5 mg/mL | 1.079 | 0.128 | - |
| 5 mg/mL | 1.080 | 0.126 | 1.08 ± 0.11, 0.12 ± 0.16 | 5 mg/mL | 1.080 | 0.126 | 1.08 ± 0.11, 0.12 ± 0.16 |

Table 4. Recovery study of *p*-coumeric and ferulic acid.

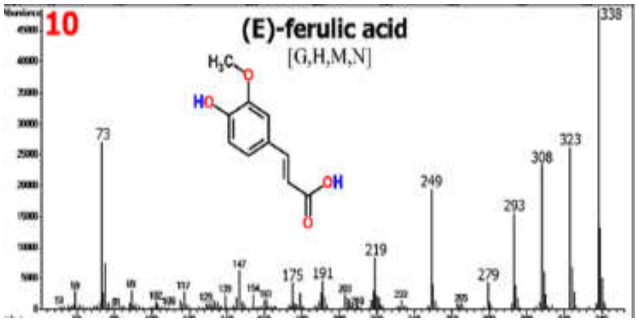
| Amount of *p*-coumeric and ferulic acid added (%) | Amount of *p*-coumeric and ferulic acid assay (%) | | % Recovery | | Mean recovery ± % RSD | |
|---|---|---|---|---|---|---|
| | | | | | *p*-Coumeric | Ferulic acid |
| Un-spiked | 1.08 | 1.58 | - | - | - | - |
| Un-spiked | 1.10 | 1.57 | - | - | - | - |
| Un-spiked | 1.09 | 1.59 | - | - | - | - |
| Spiking 10 % | 3.91 | 1.91 | 107.95 | 107.95 | - | - |
| Spiking 10 % | 3.90 | 1.90 | 108.52 | 108.52 | - | - |
| Spiking 10 % | 3.92 | 1.92 | 109.70 | 109.70 | 108.72 ± 0.81 | 108.72± 0.81 |
| Spiking 20 % | 4.23 | 2.23 | 108.68 | 108.68 | - | - |
| Spiking 20 % | 4.23 | 2.23 | 109.12 | 109.12 | - | - |
| Spiking 20 % | 4.25 | 2.25 | 110.53 | 110.53 | 109.44 ± 0.88 | 109.44± 0.88 |
| Spiking 30 % | 4.51 | 2.51 | 102.90 | 102.90 | - | - |
| Spiking 30 % | 4.50 | 2.50 | 103.19 | 103.19 | - | - |
| Spiking 30 % | 4.50 | 2.50 | 101.83 | 101.83 | 102.64 ± 0.69 | 102.64± 0.69 |

Table 5. LOD and LOQ.

| Signal to noise | Percentage | Average | LOD (% * 3.3) | LOQ (% * 10) |
|---|---|---|---|---|
| Run-1 | 0.0002 | 0.00018 | 0.002 | 0.001 |
| Run-2 | 0.0002 | 0.00019 | 0.001 | 0.002 |
| Run-3 | 0.0003 | 0.00019 | 0.001 | 0.002 |




*p*-Coumeric acid




Ferulic acid

Figure 4. ESI mass spectra of purified compounds by preparative TLC.

## CONCLUSION

In this study, a robust and simple HPLC method was developed for the quantitative determination of *p*-coumeric and ferulic acid in *Cynodon dactylon* (L) Pers. herb sample and extract forms. The mobile phase 5% acetic acid in water and acetonitrile exhibited superior chromatogram with desired shapes of the peaks. The retention times obtained in this study are lesser and easy to recover the isolates. The LOD and LOQ were found to be low with acceptable mean recovery %. The methods developed are simple, sensitive and statistically validated for linearity, accuracy and precision. The further study of NMR; IR; *in vitro* (chemical study) and *in vivo* (animal study) study will be carried to demonstrate the therapeutic activates of two major bioactive compounds.

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