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SIMULTANEOUS ESTIMATION OF PHENOLIC ACIDS AND FLAVONOIDS IN THE LEAVES OF ANAPHALIS LAWII BY USING RP-HPLC METHOD

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ABSTRACT

A RP-HPLC method with gradient elution and UV-Vis detector was develop to estimate and quantify the phenolic acids (Gallic acid, Ferulic acid, Caffeic acid) and flavonoids (Rutin, Quercetin) in the leaves of Anaphalis lawii. The developed HPLC conditions led to an efficient separation of Gallic acid (Rt=5.4), Caffeic acid (Rt=9.4), Rutin (Rt=10.4), Quercetin (Rt=12.2) and Ferulic acid (Rt=24.2) in the concentration of 0.098, 0.009, 0.021, 0.01, 0.001 µg/gm respectively.

KEYWORDS: Gallic acid, Ferulic acid, Caffeic acid.

INTRODUCTION

Anaphalis lawii (**Hook.f.) Gamble** is a wide spread, very white and tall herb belonging to the family Compositae. It is commonly known as south Indian pearly everlasting, Kalthamarai in Tamil, Tella swarupi in Telugu. It is distributed in Western Ghats, Coorg, Bababudan hills of Karnataka, Brahmagiris, hills of Coimbatore, hills of Tirupathi, Nilgiris, Anamalais, Pulneys and hills of Tinnevelly at 5000-7000 ft. Leaf margins flat, not folded back except the upper once of the scape, which are closely pressed and ascending; leaves linear oblong or oblanceolate, very white-wooly, 1-3.5 inch long, 0.3 inch broad; heads 0.2-0.3 inch broad, in broad corymbs of many branches; bracts white, limb ovate, acute; achenes minute. The whole plant is air-dried, powdered and used for the treatment of wounds and cuts by tribes in the

tirumala hills of Andrapradesh. It is also consumed with food as Kayakalpa by the Malasars of the Velliangiri hills in the Western Ghats of Nilgiri Biosphere.[1,2]

The Preliminary phytochemical studies revealed that the plant contains Phenolic compounds, alkaloids, tannins and traces of fixed oils. The total phenolic content and flavonoidal content of the plant was estimated in methanolic extract of the leaves further confirms the presence of phenolic compounds. However, there were no reports on identification and quantification of of phenolic acids (Caffeic acid, Ferulic acids) and flavonoids (Rutin, Quercetin) by using RP-HPLC with gradient elution technique. Therefore, the present study was undertaken for the quantification and determination of polyphenols in the leaves of Anapahlis lawii for the first time.

Many Epidemiological studies found that the Phenolic acids possess antioxidant, anticancer, antimutagenic, antiulcer, Antidiabetic, antiaging, aniviral, antimicrobial and hepatoprotective properties and also, numerous pharmacological effects for flavonoids, such as anti-inflammatory, antihepatotoxic, antirheumatic, antitumor, antimicrobial, antiviral, antiallergic, treatment of cardiovascular diseases and also exhibit enzyme-inhibiting activities by providing many health benefits to human RP-HPLC is the method of choice for the analysis of phytoconstituents among the various chromatographic techniques which does not require derivatization and thus it reduces the consumption of time compared to GC-MS.

EXPERIMENTAL

Chemicals and Standards

The plant leaves of Anaphalis lawii were collected from the Talakona forest near to Tirupati and were authenticated by Dr.K.Madavachetty, S.V.University, Tirupati, Andhra Pradesh in month of March 2013. The Standards of phenolic acids (gallic, ferulic and caffeic acid), flavonoids (Rutin and Quercetin) were purchased from Sigma-Aldrich (India). The solvents like Methanol (HPLC gradient grade) and Acetic acid (HPLC grade) were purchased from Merck (India).

Extraction

The leaves were separated from plants, washed well to remove adhering matter, dried under shade and powdered using blender. 100 gram of powdered leaf material was extracted in a Soxhlet with methanol. The extracts were filtered through 4-fold muslin cloth followed by Whatman No. 1 and concentrated in vacuum under reduced pressure producing the crude

extract (6.20g) and dried in the desiccators.[3,6] The plant were extracted and analyzed in triplicate. Data were reported as standard error of the mean. The values of $P \leq 0.05$ were considered to be statistically significant.

Purification of extract

The crude methanolic extract was adjusted with water to give 90% and then partitioned with t-Butylmethyl ether and n-hexane mixture (9:1) in separating funnel. It was then shaken very well and the methanolic portion was separated, evaporated and used for chromatographic techniques.

Sample Preparation

The separated extracts were subjected to column chromatography and the obtained fractions are studied for the presence of polyphenols by TLC. The isolated fractions which show positive results for the presence of flavonoids were evaporated to dryness at 50^{o}C. It was then reconstituted with 10 ml of mobile phase (**Solvent A** - Water - Acetic acid (25:1); **Solvent B** – Methanol) and filtered in a 0.45 mm membrane filter paper (Millipore). The 20 µL of the sample solution was then injected into HPLC system.[7,8]

HPLC Equipment

HPLC (**Shimadzu CLASS-VP V6.14 SP2)** analyses were performed with dual pumping system (LC-10ATVp) and SCL 10A system controller including variable Shimadzu SPD-10ATVp UV VIS detector operated at 280nm and a sample injection valve with a loop size of 20 µl. The separation was carried out by using C18 reversed phase HPLC column (5 µm particle size, i.d. 4.6 x 250 mm) at 28^{o}C.

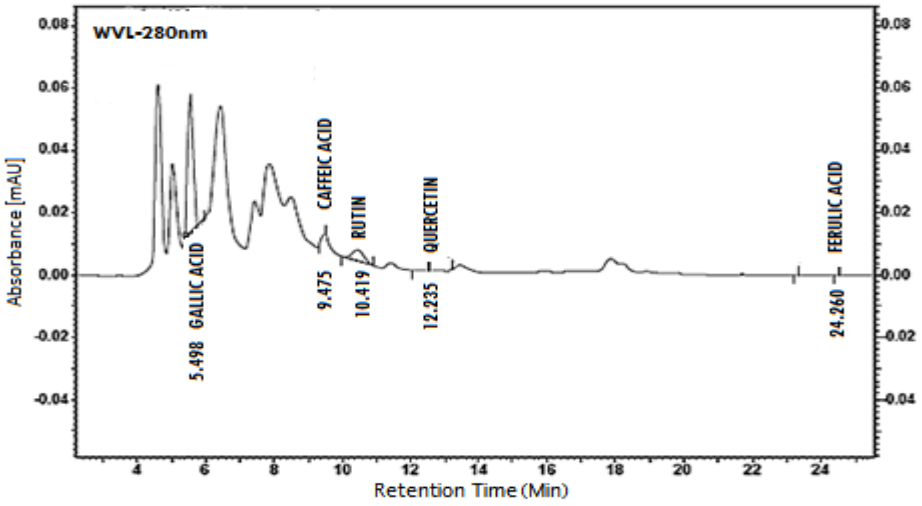
HPLC Analysis of phenolic acids and Flavonoids

The gradient elution of mobile phase consists of solvent A [water-acetic acid (25:1 v/v)] and solvent B (methanol) plays a significant effect on the effective resolution of compounds. It was carried out by varying the proportion of Solvent A to Solvent B. There is subsequent increase in Solvent B to 10% in 4 min, from 10% to 50% in 10 min, from 50% to 80% in 30 min. The composition of mobile phase back to the initial condition in 35 min with a constant flow rate of 1.0 mL/min. HPLC chromatograms were detected using a UV detector at 280nm according to absorption maxima of analysed compounds. The quantification of the phenolic acids and flavonoids in the sample was done by measuring the integrated peak area with retention time (Rt) of the respective standard (Rutin, Quercetin, Gallic Acid, Ferulic Acid and

Caffeic acid) and further confirmed by co-injection of samples with isolated standards. The peak area was calculated with the help of CLASSVP software. The content of each compound in the sample was calculated from the corresponding calibration curve of respective standards. The data were reported as means ± standard deviations in triplicates.

RESULTS AND DISCUSSION

RP-HPLC analysis is the most common method used for the separation and identification of plant metabolites. The chromatographic method developed for the identification and quantification of polyphenols provides a quick analysis of the methanolic extract. The developed HPLC conditions led to an efficient separation of peaks which could be easily identified in chromotagram (Figure 2) as Gallic acid (Rt=5.4), Caffeic acid (Rt=9.4), Rutin (Rt=10.4), Quercetin (Rt=12.2) and Ferulic acid (Rt=24.2). The content of each compound were calculated from the corresponding calibration curve and presented in Table 2. The obtained values were identified by comparing the retention time of 5 standard reference compounds as Gallic Acid (Rt=5.75), Caffeic acid (Rt=9.45), Rutin (Rt=10.51), Quercetin (Rt=12.40) and Ferulic acid (Rt=24.17) (Figure 1 and Table 1).



Retention Time (Min)	Area %	Height %	Concentration (µg/gm)	Name
5.4	80.15	90.05	0.098±0.003	Gallic acid
9.4	1.22	1.38	0.009±0.005	Caffeic acid
10.4	14.54	7.52	0.021±0.02	Rutin
12.2	0.08	0.19	0.01±0.003	Quercetin
24.2	0.02	0.01	0.001±0.04	Ferulic acid

Values are obtained by calculating the average of three experiments and data are presented as Mean ± SEM. P<0.05

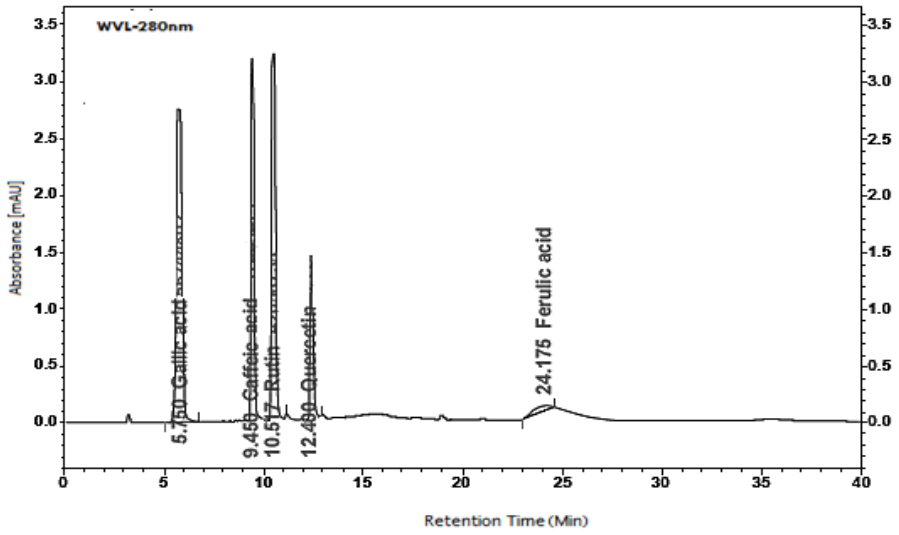


Figure 1: HPLC chromatogram of Standard Gallic Acid, Caffeic Acid, Rutin, Quercetin, Ferulic Acid.

Retention Time (Min)	Area %	Height %	Concentration (µg/ml)	Name
5.75	42.80	29.26	10	Gallic acid
9.45	13.61	20.35	10	Caffeic acid
10.51	31.78	34.52	10	Rutin
12.40	10.29	14.37	10	Quercetin
24.17	2.10	0.40	10	Ferulic acid

CONCLUSION

The RP-HPLC method with UV-Vis Detector for the quantitative estimation of Phenolic acids and flavonoids in the methanolic extracts of Anaphalis lawii was developed. It is worthy to mention that the polarity increases the quantification of highly polar phenolic acids and also flavonoids. They are detected even in low concentration in the polar solvent (methanol extract). However, the extract in 80% methanol (Solvent B) is found to be the optimum solvent of choice as it contains the maximum separation of bioactive components. The obtained results showed that the leaves of Anaphalis lawii contains considerable amount of biologically active phenolic acids and flavonoids, which proves their medicinal benefits by performing animal studies. The present study ensures unambiguous recommendation of the plant Anaphalis lawii for the use in pharmaceutical sectors as it possess potent

pharmacological activities and also suggests for the promotion of various health benefits to humans.

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