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DETERMINATION OF ANTIOXIDANT CONTENT OF FEW AROMATIC PLANTS BY RP-HPLC METHOD

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ABSTRACT

A rapid, simple and precise high-performance liquid chromatographic (HPLC) method coupled with photo diode array detector was developed for the simultaneous determination of gallic acid, catechin and caffeic acid in aromatic tulsi plant. The chromatographic method used Micrbondapak C18 column as the stationary phase, 0.2% ortho phosphoric acid in water and acetonitrile at a ratio (81:19) as the mobile phase. The flow rate was maintained at 1.2 ml/min and wavelength of detection was set at 225 nm. The calibration curve showed good linearity with regression coefficient of 0.998, -0.998-0.997 respectively for gallic acid, catechin and caffeic acid in the linear concentration range of 1.6 µg/ml to 25.6µg/ml. The relative standard deviation of intra-day and inter-day precision for three the analytes was found to be less than 3% and a very good separation of the components was obtained with retention time of 3.22, 4.92 and 6.89 respectively. The d was successfully appliegd to the estimation of caffeic acid, catechin and gallic acid inmethanolic extracts of different species of tulsi plant (Ocimum sanctum and Ocimumtenuiflorim) using the newly developed HPLC method.

KEYWORDS: Ram tulsi, Krishna tulsi, HPLC, Caffeic acid, catechin, gallic acid.

1. INTRODUCTION

Antioxidants are compounds capable of preventing or reducing harmful effects of free radicals, which may cause a broad range of diseases like inflammation, stroke, heart disease, diabetes mellitus, cancer, Parkinson's disease and Alzheimer's disease. Antioxidant constituents of plants act as radical scavengers and help in converting the free radicals to less reactive species. Most naturally occurring antioxidants of plants are flavonoids, vitamins, phenols, carotenoids and dietary glutathiones. These plant derived antioxidants have been reported to quench singlet oxygen, scavenge free radicals, inhibit enzymes and decompose peroxides.[1-19] Phenolic compounds constitute a large group of secondary plant metabolites called polyphenols (flavonoids and tannins) and simple phenols (phenolic acids and coumarins). Phenolic acids are hydroxylated derivatives of benzoic and cinnamic acids and the most common among them are caffeic acid, gallic acid, p-coumaric acid, ferulic acid, p-hydroxybenzoic acid, vanillic acid and procatechuic acid which frequently occur in fruits and vegetables as esters or glycosides. Catechins are flavonoids which are often investigated due to their health-promoting properties, anti-mutagenicity, anti-obesity, antibacterial, lipid lowering and bowel modulating action. Most of these functions have been attributed to the anti-oxidation and free radical scavenging activities of catechins.[20-38]

Tulsi or Holy basil is a medicinal plant belonging to the family *Lamacaea.* It is used to treat common health issues in traditional medicine. Tulsi plant especially leaf part have been used for skin problems, common cold, cough, headache, indigestion etc and shows good anti-inflammatory activity. The reason for pharmacological activities exhibited by the plant is due to the chemical constituents and secondary metabolites present in it. There are two types of tulsi the first one is called as Krishna tulsi (*Ocimum sanctum*) – Has purple leaves and Ram tulsi (*Ocimumtenuiflorim*) – Has Green leaves. The chemical constituents present in the plants may vary depend upon different species.[39]

A prerequisite for investigating the biochemical effect of plant species is to know its qualitative and quantitative composition. The content of phenolic compounds in plant samples can be determined by various analytical instrumental methods, such as gas chromatography, thin-layer chromatography, and capillary electrophoresis. However, high performance liquid chromatography (HPLC) has proved to be the most appropriate owing to the structural similarity and diversity of phenolic

compounds, allowing the analysis with sufficient precision, selectivity and within a reasonable time.[40-54] Therefore, in this work a HPLC method was developed and validated to characterise few phenolic compounds viz., caffeic acid, catechin and gallic acid in the methanolic extract of Tulsi plant and to study the variation of contents in two varieties of the plant.

2. MATERIALS AND METHOD

2.1. Plant material

Fresh plant leaves were collected from Krishnagiri, Tamilnadu, India and used for the study. The samples were cleaned and dried at room temperature under shade for three days. The dry samples were powdered well prior to the methanolic extraction.

2.2. Reagents

HPLC grade acetonitrile and ortho-phosphoric acid (AR) were used for the preparation of mobile phase. HPLC grade methanol was used for the extraction procedure. HPLC grade water was prepared by using Millipore MilliQ water purification system. Standard marker compounds of caffeic acid, catechinand gallic acid were obtained from Sigma-Aldrich.

2.3. HPLC-UV/DAD analysis

Chromatography was performed on Shimadzu LC-20 AT HPLC connected with SPD-M20 A Prominence diode array detector along with mannual injector. The analysis was carried out on an Mirobandapak C18 column (150mm×4.6mm, 5µm) along with guard column(10mm ×4mm) the mobile phase was composed of acetonitrile and water in the ratio of 19:18. The flow rate was 1.2 ml/ min. The samle injection was 20µL and ambient temperature was maintained throughout the analysis.

2.4. Preparation of the extracts.

For the process 2 grams of dried powdered leaves were socked in 50 ml of HPLC grade methanol and sonicated for 20 minutes. The methanolic part was replaced with fresh methanol and set aside for one day. The combined extract was filtered through Whatmann filter paper no 1 and it were evaporated to dryness at room temperature. The dried extract was dissolved in 2 ml of methanol and stored at 4 ºC prior to the use.[55]

2.5. HPLC method validation

Validation of the HPLC method was performed in agreement with international guidelines for analytical tachniques for the quality control of pharmaceuticals (ICH guidelines)[56] The stock standard solutions were prepared by dissolving 10 mg of caffeic acid, catechin and gallic acid in 10 ml of methanol in separate standard flask. The standard solutions were mixed together and diluted with mobile phase to obtain a seriese of standard solution with concentarions of 1.6, 3.2, 6.4, 12.8 and 25.6 µg/ml. Further, 20µL aliquots of each standard solution were used for the HPLC analysis; the injections were performed in triplicate for each concentration level. The calibration curve was obtained by plotting the peak area versus the concentration. The content of caffeic acid, catechin and gallic acid were determined by regression equation using the calibration curve.

Limit of detection (LOD) and the limit of quantification (LOQ) were experimentally determined by using regression co-efficient. The accuracy of the analytical procedure was evaluated by the recovery experiment. This involved the addition of a known quantity of standard compound to half the amount of a methanolic extract of leaf extract to reach 100% of the test concentration. The spiked samples were then analyzed with the proposed method and precision of the chromatographic system was tested by performing intra-day and inter-day multiple injections of a methanolic extract and then checking the %RSD of retention time sand peak areas. Six injections were performed on each day and carried out for three consecutive days.

3. RESULT AND DISCUSSION

3.1 Optimization of chromatographic conditions

To obtain a good chromatographic separation with better resolution of components within shorter analysis time, the chromatographic conditions were optimised. The resolution of these compounds were tested and compared with different ratios of mobile phase. Because of low wavelength threshold of absorption compared with methanol and better resolution acetonitrile was fixed as the organic phase. Considering the nature of the compounds acidic condition was preferred for the mobile phase. Hence, 0.2% ortho-phosphoric acid of HPLC grade in water was used as the aqueous phase. For better resolution the column temperature was set at 20º -25 ºC. By considering the absorption maxima of three compounds on the UV spectra in three-dimensional chromatogram mode of HPLC-DAD detection, the wavelength was set at 225nm for gallic acid, catechin and caffeic acid.

3.2 Validation of HPLC Method

3.2.1 Specificity

The specificity was assessed by comparing the chromatogram obtained from the analysis of a blank sample, standard solution and sample solution. The peak in the chromatogram of the sample solution corresponded in retention time to the peak in the chromatogram of the standard solution. No peak of sample and standard appeared at the retention time of the solvent. This indicates that the method was specific for the analytes under consideration.

3.2.2 Linearity

Series of standard solutions of three compounds were freshly prepared in mobile phase and used to determine the linear range of the analytes. The results obtained for the calibration curve is given in Table 1. Good linear relationship and correlation coefficient were achieved. The limit of quantification (LOQ) and limit of detection (LOD) values of individual compound clearly indicated

that the analytical method was acceptable with excellent sensitivity.

3.2.3 Accuracy

For the accuracy of proposed method, recovery studies were performed by standard addition method at three different levels (50%, 100% and 150% of final concentration). A known amount of standard pure drug was added to the pre-analyzed herbal extract and the sample was then analyzed by the proposed method. Results of recovery studies were found to be satisfactory and given in the Table 2.

3.2.4 Precision

The intraday and inter day variability of precision was analysed by replicates at three different concentration search day on three consecutive days. The RSD of intra-day and inter-day variability of precision were in the range of prescribed values.

3.2.5 Limit of detection and limit of quantification

The LOD is the lowest quantity or concentration that can be detected by the given analytical method (LOD=3.3× D/S) and The LOQ is the smallest concentration of the analyte, which shows response that can be accurately quantified and LOQ = 10 × D/S where, D is the standard deviation of y – intercepts of regression line and S is the slope of the calibration curve.

The two samples-extracts of Krishna tulsi and Ram tulsi were analysed using the new method and the content of phenolic acids calculated using regression equation method. The results are shown in the table.

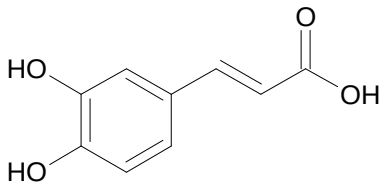

Fig. 1: Caffeic acid.

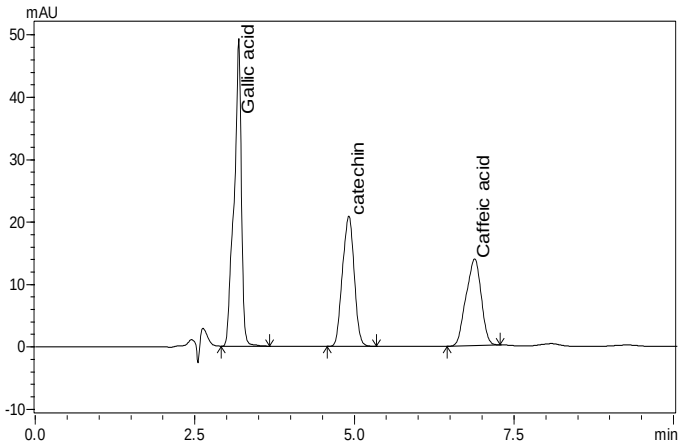

Fig. 2: Chromatogram of standard mixture of Gallic acid, Caffeic acid and Catechin.

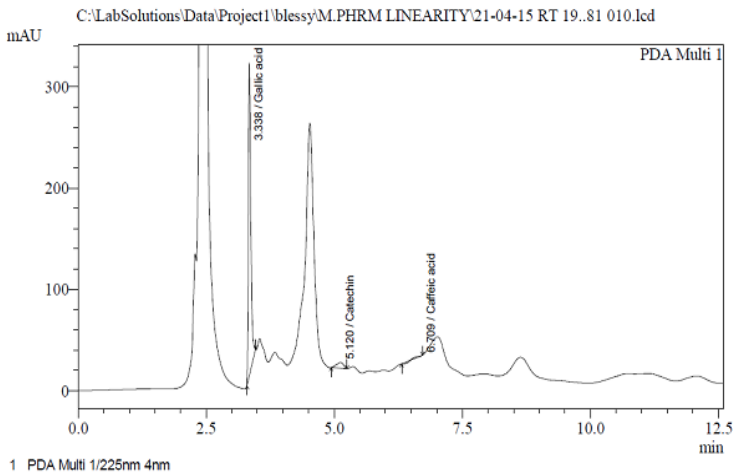

Fig. 3: Chromatogram of Ram tulsi extract.

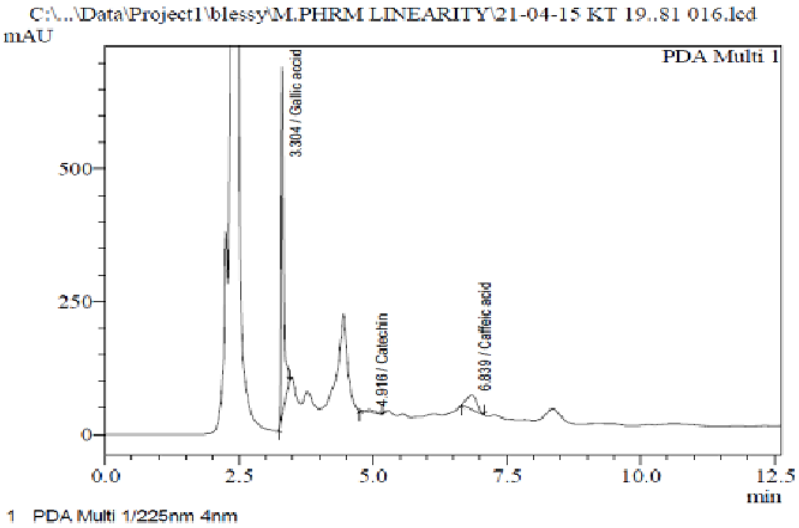

Fig. 4: Chromatogram of Krishna tulsi extract.

Table 6: Quantitation of caffeic acid, catechin and gallic acid in the samples of Krishna Tulsi and Ram Tulsi.

Components	Caffeic acid (mg/100mg)	Catechin (mg/100mg)	Gallicacid (mg/100mg)
Krishna tulsi	0.263323	0.034692	1.6150
Ram tulsi	0.001153	0.032074	0.5795

4. CONCLUSION

A validated and sensitive HPLC method with high precision, robust and repeatability was developed for the simultaneous quantitation of three phenolic compounds – Gallic acid, Catechin and Caffeic acid in aromatic tulsi plants. The new method is accurate, precise and rapid as the analysis time is less than 15 minutes and the method were successfully applied to tulsi samples. The peaks for the phyto-constituents were identified by comparison with the UV spectra and retention time of the standard compounds.

Krishna tulsi was found to contain more amounts of caffeic acid and gallic acid compared to Ram tulsi. To be exact that Krishna tulsi contains more phenolic acids than other species.

There is no much variation in catechin content among the two verities.

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