Development of HPLC method by UV-VIS detection for the quantification of phenolic acids in different *Ocimum sanctum* Linn. extracts

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Abstract: A simple and rapid chromatographic method has been developed for the simultaneous determination of five phenolic acids including Gallic acid, Chloroganic acid, Syringic acid, Benzoic acid and Vanillic acid by HPLC with UV-VIS detector. These Phenolic acids were separated by analytical column Intersil ODS-3 C18, a gradient elution system of ACN and acidified water solution with 1ml/min flow rate and quantified in a total run of 30 minutes at 210nm wavelength. In the quantitative analysis of these compounds showed good regression (0.995-0.999). The limit of detection [LOD] and limit of quantification [LOQ] of these compounds were in the range of 0.15-0.46 and 0.42-2.47 µg/ml. The average recoveries were between 95.8–103.1% and their RSD values were less than 3.34%. By the proposed method Gallic acid, Chloroganic acid and Syringic acid were found and quantified in Methanolic, Ethanolic and Acetonic extract of *Ocimum sanctum* Linn. leaves. While the two other phenolic acids benzoic acid and vanillic acid was not found in the extracts of *Ocimum sanctum* Linn. leaves.

Keywords: Phenolic acids, method validation, extraction, *Ocimum sanctum* Linn.

INTRODUCTION

Plants and plant extracts are widely used as traditional medicine for curing various diseases because of therapeutic potentials. The pharmacological studies have shown that plants have active components that show antioxidant, antiviral, anti-inflammatory, antitumor and antimicrobial activities.(Rolim *et al.,* 2005; Abdel-Hameed 2009). The antioxidant activity in plants is due to the presence of phenolic (biologically active non nutrient) substances (Rice-Evans *et al.,* 1996) which have antioxidative and anticarcinogenic effects (Manuel *et al.* 2006). The phenolic contents are the most important source of potential health promoting photochemicals that can be divided into many categories including flavonoids, flavonols, flavones, isoflavonoids, catechins and phenolic acids. (Kliebenstein 2004). Phenolic acids are the hydroxylated derivatives of benzoic and cinnamic acids (Sundaram 2012) which are present in plants in free and in bond forms. The bonded phenolic may be linked to various plant components through acetal, ester, or ether bonds. (Yilmaz *et al.* 2009; Iş & Demir 2012). The daily consumption of phenolic acids estimated by (Mattila & Hellström 2007) ranged from 25 mg to 1 g. Phenolic acids and flavonoids having many functions in plants. Importantly, they protect a cell wall and help in seed dispersal and pollination due to color attractants for birds and insects. It also provides help in the defensive mechanisms of plants in infections like wounding and UV irradiation or excessive light. In the same vein, it was proofed that Flavonoids possess antiviral, anti inflammatory, anti allergic and anti proliferative activities (Şahin *et al.,* 2009; Luo *et al.,* 2008). The published data on phenolic acid profiles of plant extracts are still incomplete and complicated. Therefore, There are different Chromatographic techniques to analyze phenolic acid in plant extracts, such as thin layer chromatography (TLC) high performance liquid chromatography (HPLC), ultra high performance liquid chromatography (UHPLC) and gas chromatography (GC) (Sharma & Patel 2009; Peng *et al.,* 2008; Stalikas 2007). These techniques are found useful for drug analysis because by using these techniques a small amount of samples required, are sensitive, reproducible and reliable but time consumable and cannot be applied for the characterization in plant extract.

The purpose of the current study was to develop a simple and rapid HPLC method to determine the composition of phenolic acids in plant extracts. This can be used for assessment of phenolic acid in herbal formulations. This is the most popular technique as compare with other analytical techniques used for detection and quantification. It has many advantages like fast analysis, isolation of compounds, a small amount of sample is required for qualitative and quantitative analysis and highly sensitive and accurate (Padda & Picha 2008; Scalbert *et al.,* 2005). The newly reported method was

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utilized to analyze the targeted phenolic acids in different extract of *Ocimum sanctum* Linn. plant.

MATERIALS AND METHODS

Apparatus

The analyses of phenolic acids were carried by HPLC (Hitachi D-2000 Elite system manager) equipped with two pumps (Models L-2130), auto-injector L-2200 syringe loading sample injector valve's fitted with 20µl sample loop of 200 vials and UV-VIS detector L-2420. The Chromatographic separation was achieved using Column oven L-2300 and analytical column intersil ODS-3 C18 (GL Sciences Inc. Tokyo Japan 5µm, 250×4.6 mm). filterting assembly (Model Rocker-300 Thaiwan) and ultrasonic cleaner Ceia (Model CP-104 Italy) were used for solvents filtration and degassing.

Chemicals and reagents

All chemicals were of analytical grade. Gallic acid, Chloroganic acid, Benzoic acid Vanillic acid, Syringic acid were obtained from Sigma (Aldrich Germany), Methanol, Acetonitril, orthophosphoric acid and diethylamin of HPLC grade (Sigma Aldrich Germany) and Monopotassium phosphate and citric acid Extra pure (Scharlau Spain). Deionized water and Solvents of LC grade was obtained by filtering through 0.45µm filter membrane and degass for 20 minutes by ultra sonic cleaner.

Chromatographic conditions

The mobile phase of HPLC was optimized to obtain the desired phenolic acids. The mixture (v/v) of ACN (solvent A) and 0.1% aqueous acidic acid solution at 3.0 pH (solvent B), proven to be a best optimal mobile phase system in the following gradient elution program for the purpose of chromatographic separation. 0 min, 10%A-90%B; 5min 20%A-80%B: 10min 40%A-60%B; 15min 60%A-40%B; and 20min 80%A-20%B. the chromatograms were obtained at a UV wavelength of 210nm as all tested components have major absorption at this wavelength the flow rate was set at 1.0 ml/min for optimal separation and column temperature was kept at 25°C and the Identification of each phenolic acid based on retention times and on spectral data in comparison with authentic standards and quantified by establishing calibration curve of each compound standard.

Preparation of extracts

The collected plant materials were washed with distilled water, chopped into small pieces, air dried and grinded into powder by the help of grinder. 20g powders were extracted with 95% ethanol, 99.8% methanol and 99.7% acetone at 35-40°C for 1 week using cold extraction method (Şahin *et al.,* 2011) and concentrated by rotary evaporator. 0.5g of dry residues was dissolved in 10ml ACN and centrifuge at 5000rpm for 10 minutes. The clear supernatant was then filtered through 0.45um cellose (Millipore) to inject HPLC.

Validation of quantitative analysis

Standard stock solution of Gallic acid, Chloroganic acid, Syringic acid, Benzoic acid and Vanillic acid were prepared by dissolving 0.01g in 10ml Acetonitril (1mg/ml) and further diluted to 5, 10, 15, 20, 25 and 30µg/ml for standard curve preparation. Precision was determined by injecting 10 µl of each standard solution. (*n*=6). The peak areas were automatically measured by an integrator of HPLC instrument. Calibration curve was obtained by plotting the peak area against concentration. This showed linearity in accordance to Beer's law. The limit of detection and quantification under the present chromatographic conditions were evaluated at S/N of 3 and 10, respectively. The accuracy of the method was determined for each component by analyzing the recovery in percent of *Ocimum sanctum* Linn. leaves extracts spiked with six different concentrations and the robustness was investigated by changing the flow rate, the chromatographic temperature and the detection wavelength, and found to be a fine robustness of the proposed method.

RESULTS

Regression data, LODs and LOQs of Gallic acid, Chloroganic acid, Syringic acid, Benzoic acid and Vanillic acid are given (table 1). The peak area of each phenolic acid standard at different concentration was automatically measured by an integrator of HPLC instrument. Linearity was determined by drawing calibration curves(Laghari *et al.* 2011; Politeo *et al.* 2007). For the construction of calibration curve, six calibration standard solutions (5, 10, 15, 20, 25 and 30µg/ml) was injected in triplicate. Fig. 1. A linear equation was drawn between the concentration and peak area of each compound (Graph 1) as Y= mx +c here y is peak area, x is concentration while m and c are constant. A good linearity was found in the range 0.995-0.999 for all the compounds (table 2). The limit of detection (LOD) was determined by diluting standard solution of known concentration until the response was three times the noise S/N=3, which was in the range 0.01-0.1µg/ml for all the components while limit of quantification (LOQ) was calculate on the basis of minimum accepted value of S/N=10 and quantification of Phenolic acid were found in the range of 0.20-0.30µg/ml. The precision was evaluated by analyzing the peak area of each phenolic acid six times on the same sample. Precision was expressed in %RSD were in the range of 1.45-3.11% (n=6) (table 3).

In the study of the recovery of the procedure the powder *Ocimum sanctum Linn.* plant samples was spiked with each phenolic acid in a known concentration, subjected to the given extraction procedure and analyzed before and after the addition in triplicate. Recoveries for phenolic acids ranged from 95.5% to 103.1%. Relative standard deviation of recovery was in the range of 0.94 to 3.12%.

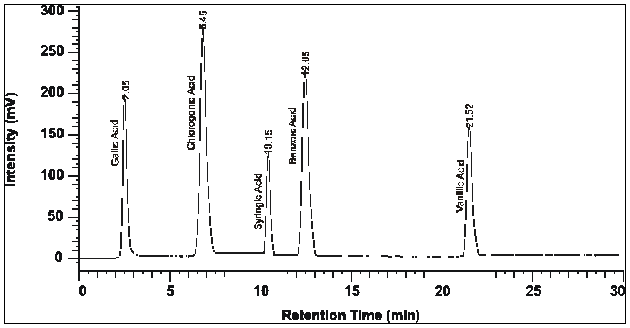

Fig. 1: HPLC Chromatogram of five mixed phenolic acids standard: The peak at retention time of 2.05 for Gallic Acid, 6.45 for Chloroganic Acid, 10.15, 12.05 and 21.52 for Syringic Acid, Benzoic Acid and Vanillic Acid respectively.

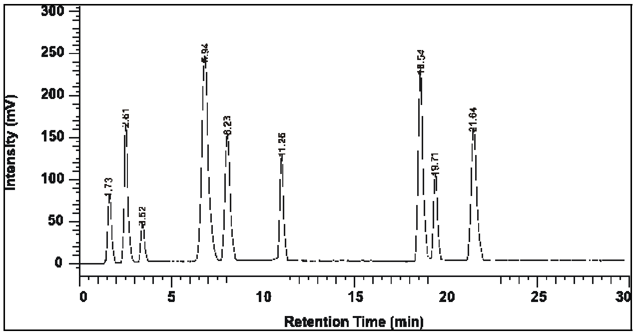

Fig. 2: HPLC Chromatogram of Methanolic extract of *Ocimum sanctum Linn.* leaves: The peaks at retention time of 2.61, 6.94 and 21.64 are for Gallic Acid, Chloroganic Acid and Vanillic acid while the other peaks are not identified.

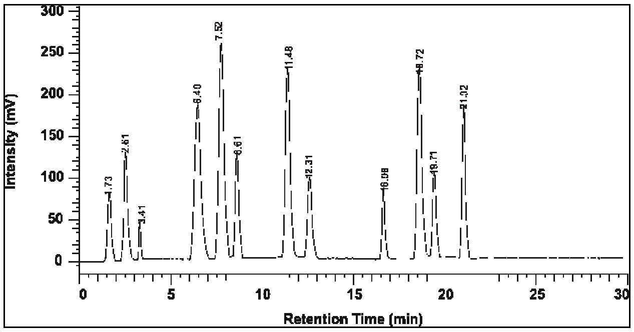

Fig. 3: HPLC Chromatogram of Ethanolic extract of *Ocimum sanctum Linn.* leaves: the peaks at retention time of 2.61, 6.40 and 21.02 is for Gallic Acid, Chloroganic Acid and Vanillic acid while the other peaks are not identified.

The proposed method applied on different extract of *Ocimum sanctum Linn.* leaves for the identification and quantification of Phenolic Acids. Figs. 2-4 (table 4) The Gallic acid found in Ethanolic, Methanolic and Acetonic extract were 3.23, 2.97, 3.13µg/ml, Chloroganic acid 1.48, 1.86, 1.12µg/ml and Vanillic acid 1.13, 1.59, 1.09µg/ml respectively. While the Syringic acid and Benzoic acid was not detected in any extract.

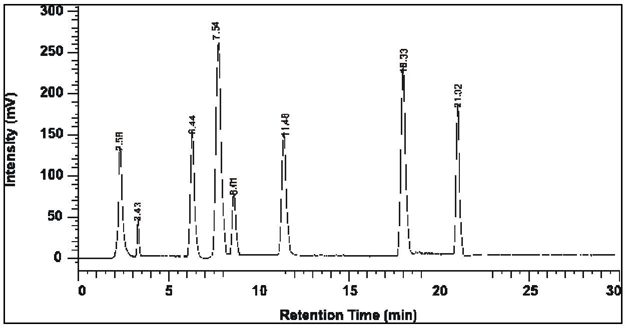

Fig. 4: HPLC Chromatogram of Acetonic extract of *Ocimum sanctum Linn.* leaves: the peaks at retention time of 2.58, 6.44 and 21.02 is for Gallic Acid, Chloroganic Acid and Vanillic acid while the other peaks are not identified.

DISCUSSION

A simple reversed phase high performance liquid chromatographic methods are generally used for the separation of phenolic acids. Such a separation are simple rapid and provide high resolution and sensitivity (Vrchovská *et al.* 2007; Umesha *et al.*). The phenolic acids detected in extracts were Gallic Acid, Chloroganic Acid and Vanillic Acid. The present study is to design an HPLC (UV-VIS) method validation for the detection and quantification of five major phenolic including Gallic acid, Chloroganic acid, Syringic acid, Benzoic acid and Vanillic acid. These entire compounds have already been described in by different detector. The best detector chooses for separation of phenolic acid was Diode Array Detector DAD (Fattouch *et al.,* 2007).

But here it is reported for the first time by the detection of UV-VIS. In method development the selection of mobile phase and column become crucial for achieving good separation. In the optimization of solvent system for the separation of phenolic acids different ratio and different mobile system are used containing acidified water and a polar organic solvent. The addition of acid improve the separation by suppressing the ionization of acidic groups (Srivastava 2009). In trail assay elution program using of acetic acid, phosphoric acid solution at different pH (3-8) and ACN and methanol were tested. However ACN and acetic acid in gradient elution program was found good for Phenolic acids separation. By the proposed method we successfully detect and quantified the five phenolic acids in *Ocimum sanctum* Linn. leaves extract. In which the Syringic acid and benzoic acids are absent in all extracts.

Table 1: Peak area of phenolic acid standards at different concentrations

S. No	Standard	Peak Area of Concentration µg/ml					
		5	10	15	20	25	30
1	Gallic Acid	17682	25455	34567	42965	51345	59432
2	Chloroganic Acid	31456	38765	45643	53235	61543	71565
3	Syringic Acid	15234	22345	31425	39265	48536	56543
4	Benzoic Acid	24356	31876	39456	45876	53806	60238
5	Vanallic Acid	29656	36765	43236	49879	58376	66567

Table 2: Parameters of linearity, detection and quantification limits of the phenolic acids

S. No	Standard	Y=mx+c Calibration Equation	Regression	LOD (µg/ml)	LOQ (µg/ml)
1	Gallic Acid	Y= 8370X + 6262	0.999	0.05	0.25
2	Chloroganic Acid	Y= 7899X + 22721	0.995	0.01	0.20
3	Syringic Acid	Y= 8408X + 9128	0.999	0.10	0.30
4	Benzoic Acid	Y= 7189X + 17439	0.999	0.01	0.20
5	Vanallic Acid	Y= 7315X + 21810	0.997	0.03	0.20

Table 3: Precision, retention time and recovery of phenolic acids

S. No	Standard	Precision RSD%	Retention Time	Repeatability RSD%	Recovery %	RSD %
1	Gallic Acid	1.45	2.05	0.87	98.4	1.34
2	Chloroganic Acid	2.23	6.45	1.08	95.8	2.76
3	Syringic Acid	1.76	10.15	0.69	103.1	3.34
4	Benzoic Acid	1.60	12.05	1.54	98.5	2.23
5	Vanallic Acid	3.11	21.52	1.85	99.65	1.69

Table 4: Quantification of phenolic acids in *Ocimum sanctum* Linn. leaves extracts

S. No	Standard	Concentration in µg/ml of Tulsi leaves Extracts		
		Ethanolic	Methanolic	Acetonic
1	Gallic Acid	3.23±0.56*	2.97±0.48	3.13±0.65
2	Chloroganic Acid	1.48±0.24	1.86±0.35	1.12±0.22
3	Syringic Acid	ND	ND	ND
4	Benzoic Acid	ND	ND	ND
5	Vanallic Acid	1.13±0.34	1.59±0.43	1.09±0.21

* Standard deviation

The Gallic acid and Chloroganic acid have already been described in *Ocimum sanctum Linn.* leaves extracts (Côté *et al.* 2010; Bueno *et al.* 2012) with the exception of Vanillic acid which is reported by the first time in the *Ocimum sanctum* Linn. leaves extracts. The other six peaks in the chromatogram of Ethanolic, seven peaks in Methanolic and three extra peaks in Acetonic extract was declared unidentified compounds.

CONCLUSION

The aim of this study was to develop a selective and sensitive HPLC method for the rapid detection and quantification of Phenolic Acids by UV-VIS detector, and found the current method is valid, rapid and accurate for the simultaneous determination of the targeted phenolic acids in plant extracts.

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