

## HPLC Profiles of Standard Phenolic Compounds Present in Medicinal Plants

\*Gupta Mradu<sup>1</sup>, Sasmal Saumyakanti<sup>1</sup>, Majumdar Sohini<sup>1</sup>, Mukherjee Arup<sup>2</sup>

<sup>1</sup>Department of Dravyaguna (Medicinal Plant Pharmacology), Institute of Post Graduate Ayurvedic Education and Research, Kolkata, India

<sup>2</sup>Department of Chemical Technology, Calcutta University, Kolkata, India

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### ABSTRACT

Many phenolic-flavonoids found in plants exhibit antipyretic, analgesic, anti-inflammatory & antioxidant properties. Twelve phenolic compounds namely Ellagic acid, Catechol, Gallic acid, Quercetin, Resorcinol, Tannic acid, Vanillin, Salicylic acid, Acetyl Salicylic acid, Benzoic acid, Phloroglucinol and Ascorbic acid were taken up for qualitative and quantitative HPLC analysis. The objective of this research was to determine the standard HPLC chromatograms of twelve prominent phenolic compounds found in medicinal plants using four mobile phases having different elution gradients and run times. Six mg of each standard compound was dissolved in 10 ml Methanol, sonicated and passed through Membrane Filter (0.45µm & 47mm diameter) before injecting 20 µl in Column: Symmetry C18 (5µm, 4.6\*250mm) & flow rate 1.0 ml/min. UV-detection at 280-360 nm was performed using 515 HPLC pumps and 2489 UV/VIS detectors. Four HPLC methods using different mobile phases were used. Methods A & B used varying ratios of Methanol and Acetic acid as solvents, the runtime being 22 and 25 minutes respectively. Similarly methods C and D used Acetonitrile and Phosphoric acid, run times being 30 and 45 minutes. All the methods resulted in similar pattern and sequence of elution of these phenolic compounds as unique chromatographs when analyzed individually and together. These HPLC fingerprints of standard phenolic compounds could be used as benchmarks for comparison during the qualitative and quantitative analysis of unknown compounds present in any plant sample. Use of multiple methods involving different phases would increase the validity and reliability of results.

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### INTRODUCTION

There has been a noticeable spurt in recent times in the number of studies aimed at characterizing the health-promoting properties of many phenolic compounds which exhibit antioxidant properties. They are useful in the treatment and management of cancer, cardiovascular and neurodegenerative diseases or for use in anti-aging or cosmetic products. Phenolic compounds are ubiquitous in plants which collectively synthesize several thousand different chemical structures and are characterized by hydroxylated aromatic ring(s). These compounds enable the plants to adapt to changing biotic and abiotic environments and provide colour, taste and health-promoting benefits to plant products. Phenolic compounds represent the most studied phytochemicals and have been widely explored as model systems in different areas of plant research<sup>[1]</sup>.

The pharmacological activities of any plant sample are due to the presence of metabolites, secondary metabolites and secretory products in it. These usually consist of the phenolic compounds, alkaloids, tannins, saponins, carbohydrates, glycosides, flavonoids, steroids, etc. Most phenolic compounds such as flavonoids, glycosides, triperinoids, flavonons, carbohydrates and anthraquinones are found distributed throughout the plant kingdom<sup>[2]</sup>. Similarly, the polyphenolic compounds most commonly found in plant extracts are the phenolic acids, flavonoids and tannins<sup>[3,4]</sup>. Flavonoids are 15-carbon compounds generally distributed throughout the plant kingdom<sup>[5]</sup>.

These compounds together with other phenolic structures of plant origin have been reported as scavengers of Reactive Oxygen Species (ROS) and are seen as promising therapeutic drugs for free radical pathologies<sup>[6,7,8]</sup>. Most flavonoidic compounds exhibit antipyretic, analgesic, anti-inflammatory, anti-arthritis, antioxidant and immuno-modulatory properties<sup>[9,10,11]</sup>. These activities of flavonoidic compounds may be due to the presence of gallic acid, ellagic acid, quercetin, tannin acid, vanillin, resorcinol, catechin, etc.

Many plant parts of medicinal herbs containing these phenolic compounds have been used for centuries as traditional treatment for many disorders. However pre-clinical studies for assessing the pharmacognostical, phytochemical, toxic and biological properties of any herbal drug are very essential before its clinical administration. In particular, the qualitative and quantitative analysis of the phenolic compounds found in any plant part following systematic scientific methodology and its comparison with standard phenolic compounds is very important for establishing its efficacy. The main objective of this research was to determine the chromatograms of standard phenolic chemical compounds which are commonly found in medicinal plant samples by High Performance Liquid Chromatography (HPLC) using four mobile phases having different elution gradients and run times. These HPLC fingerprints of standard phenolic compounds could be used as benchmarks for the purpose of comparison

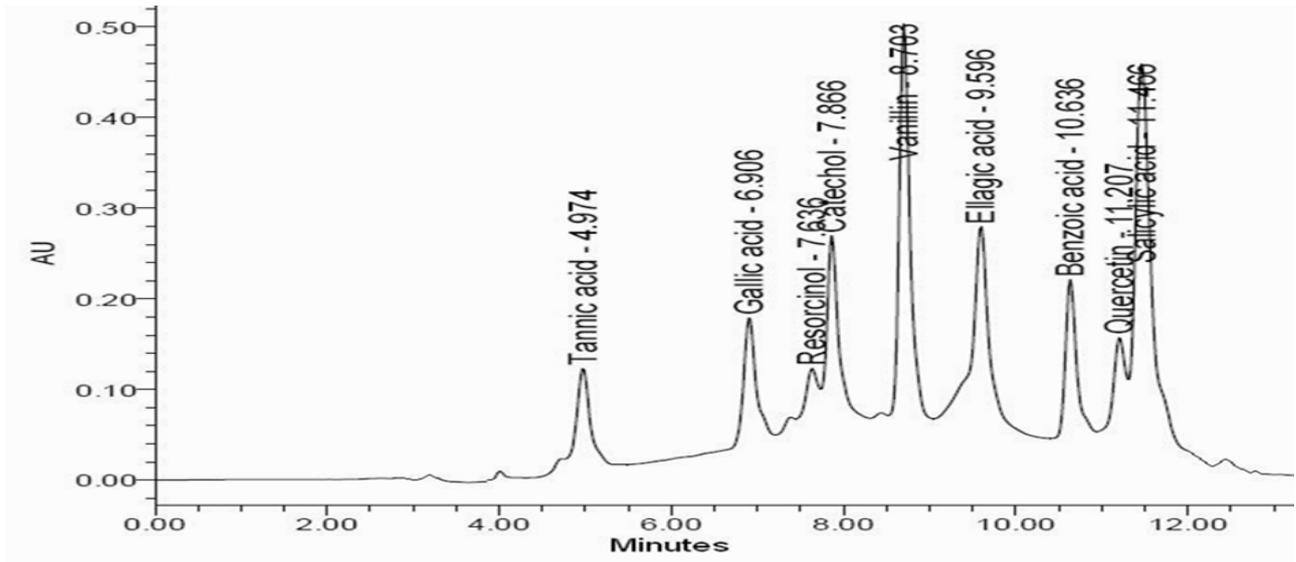


Figure 1 HPLC profile of phenolics using method A

Table 1 Retention times of phenolic peaks using method A

|    | Peak Name             | RT     | Area    | % Area | Height |
|----|-----------------------|--------|---------|--------|--------|
| 1  | Ellagic acid          | 9.596  | 6755768 | 19.24  | 268281 |
| 2  | Catechol              | 7.866  | 4004423 | 11.40  | 261734 |
| 3  | Gallic acid           | 6.906  | 4054308 | 11.55  | 174062 |
| 4  | Quercetin             | 11.207 | 1780543 | 5.07   | 141930 |
| 5  | Resorcinol            | 7.636  | 1532643 | 4.36   | 116195 |
| 6  | Tannic acid           | 4.974  | 2096906 | 5.97   | 122777 |
| 7  | Vanillin              | 8.703  | 5574723 | 15.88  | 493213 |
| 8  | Salicylic acid        | 11.466 | 6663685 | 18.98  | 443139 |
| 9  | Acetyl Salicylic acid | ---    | ---     | ---    | ---    |
| 10 | Benzoic acid          | 10.636 | 2650142 | 7.55   | 206966 |
| 11 | Phloroglucinol        | ---    | ---     | ---    | ---    |
| 12 | Ascorbic acid         | ---    | ---     | ---    | ---    |

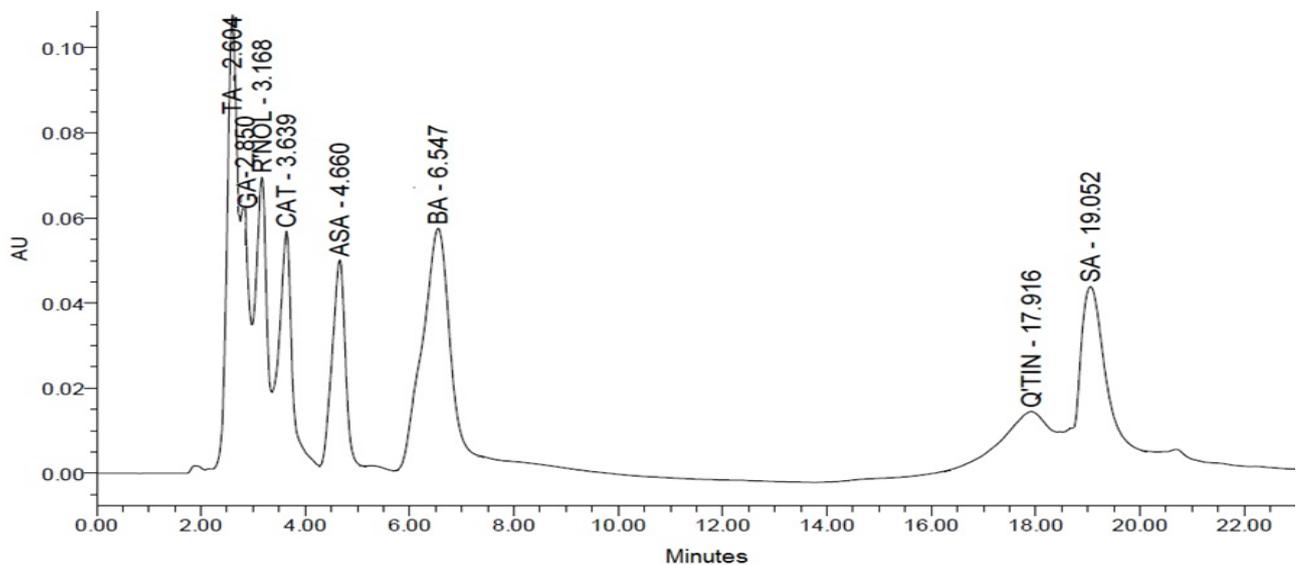


Figure 2 HPLC profile of phenolics using method B

when doing the qualitative and quantitative analysis of unknown compounds present in any plant sample. The multiple chemical constituents present in any herbal

sample could be benchmarked against these standards to assess the quality and quantity of phenolic flavonoids

Table 2 Retention times of phenolic peaks using method B

|    | Peak Name             | RT     | Area     | % Area | Height  |
|----|-----------------------|--------|----------|--------|---------|
| 1  | Ellagic acid          | 4.409  | 31616624 | 11.84  | 1109685 |
| 2  | Catechol              | 3.600  | 17636121 | 6.60   | 2286030 |
| 3  | Gallic acid           | 2.679  | 35975636 | 13.47  | 1678740 |
| 4  | Quercetin             | 18.768 | 17307027 | 6.48   | 414368  |
| 5  | Resorcinol            | 3.105  | 12228760 | 4.58   | 684827  |
| 6  | Tannic acid           | 2.477  | 38881506 | 14.56  | 3068787 |
| 7  | Vanillin              | 3.845  | 98665315 | 36.95  | 3745328 |
| 8  | Salicylic acid        | 19.512 | 5238202  | 1.96   | 103724  |
| 9  | Acetyl Salicylic acid | 4.737  | 3184124  | 1.19   | 178517  |
| 10 | Benzoic acid          | 6.752  | 5065717  | 1.90   | 119519  |
| 11 | Phloroglucinol        | 2.607  | 1255179  | 0.47   | 75685   |
| 12 | Ascorbic acid         | ---    | ----     | ---    | ---     |

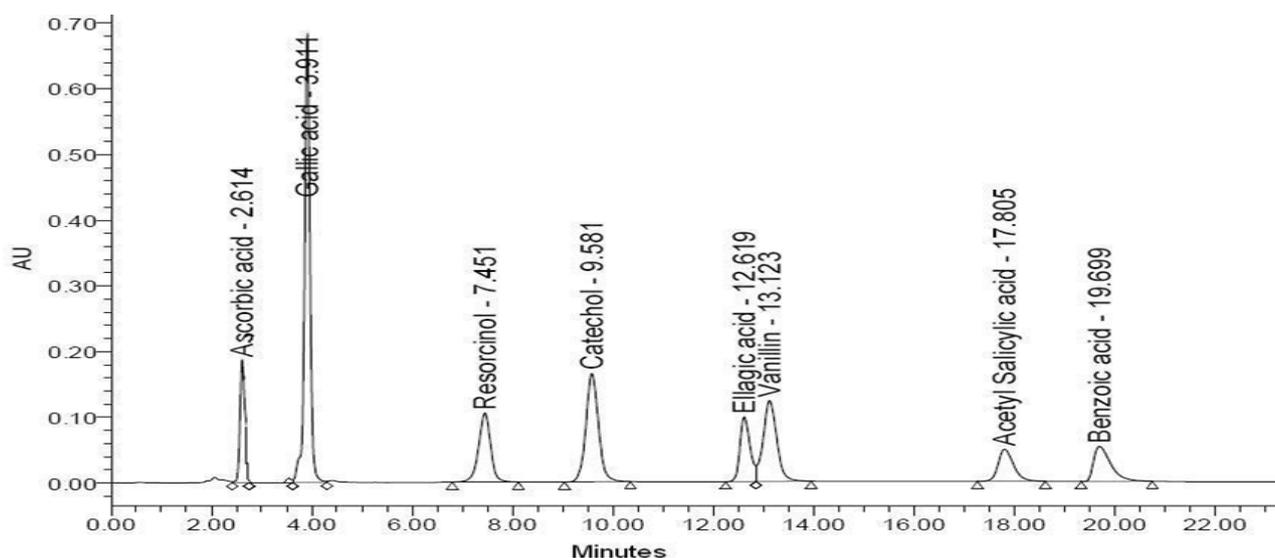


Figure 3 HPLC profile of phenolics using method C

Table 3 Retention times of phenolic peaks using method C

|    | Peak Name             | RT     | Area     | % Area | Height  |
|----|-----------------------|--------|----------|--------|---------|
| 1  | Ellagic Acid          | 11.864 | 25780029 | 13.95  | 98147   |
| 2  | Catechol              | 9.080  | 57957909 | 31.37  | 165098  |
| 3  | Gallic Acid           | 3.501  | 1920292  | 1.04   | 142380  |
| 4  | Quercetin             | ---    | ---      | ---    | ---     |
| 5  | Resorcinol            | 7.155  | 25935709 | 14.04  | 1347184 |
| 6  | Tannic Acid           | ---    | ---      | ---    | ---     |
| 7  | Vanillin              | 12.777 | 62412763 | 33.78  | 2853311 |
| 8  | Salicylic Acid        | ---    | ---      | ---    | ---     |
| 9  | Acetyl Salicylic Acid | 17.461 | 2472612  | 1.34   | 107282  |
| 10 | Benzoic Acid          | 19.196 | 5839342  | 3.16   | 239261  |
| 11 | Phloroglucinol        | ---    | ---      | ---    | ---     |
| 12 | Ascorbic Acid         | 2.564  | 2453126  | 1.33   | 291541  |

actually present, thereby giving a clear indication of its likely therapeutic efficacy<sup>[12]</sup>.

The HPLC analysis of 12 standard phenolic compounds namely Ellagic acid, Catechol, Gallic acid, Quercetin, Resorcinol, Tannic acid, Vanillin, Salicylic acid, Acetyl Salicylic acid, Benzoic acid, Phloroglucinol and Ascorbic acid was performed at the same wavelength, flow rate & sample concentration using four different methods, each having a different solvent system, gradient elution & run time. The advantage of this method is that during analysis of any research drug having phenolic

compounds, the results can be compared by using four different solvent systems, making the comparison even more stringent and reliable.

#### MATERIALS AND METHODS

The following methodology was used for obtaining the chromatogram of each standard phenolic compound as well as a mixture of all the phenolic compounds. High Performance Liquid Chromatography (HPLC) analysis was performed using four different gradients of mobile phase in different run times.

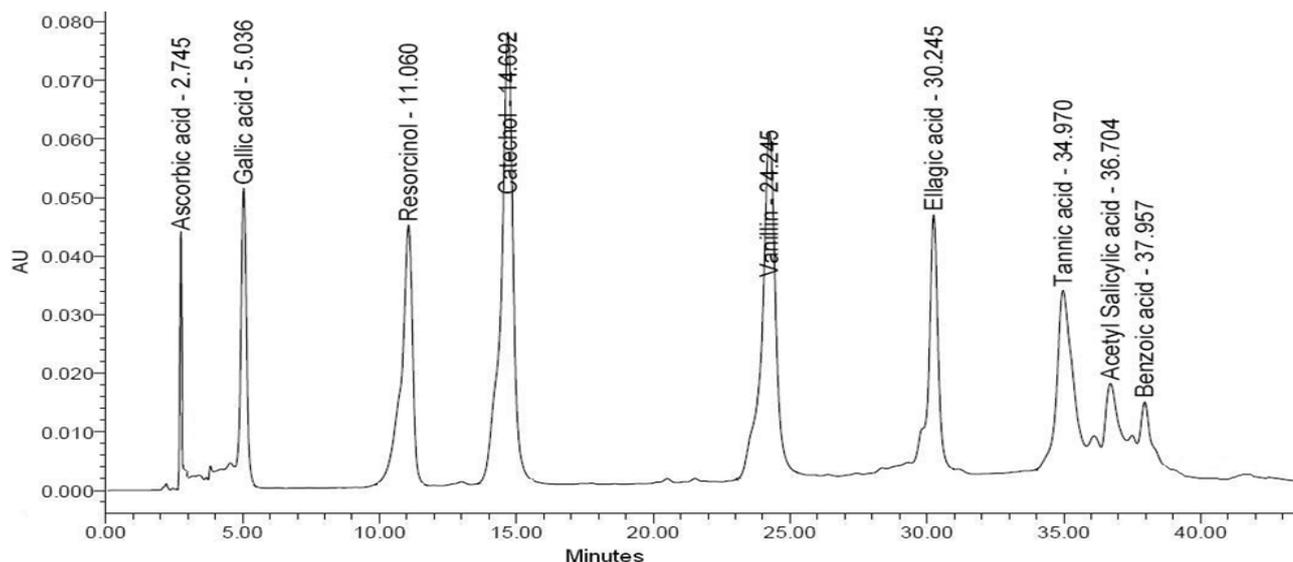


Figure 4 HPLC profile of phenolics using method D

Table 4 Retention times of phenolic peaks using method D

|    | Peak Name             | RT     | Area     | % Area | Height  |
|----|-----------------------|--------|----------|--------|---------|
| 1  | Ellagic acid          | 31.889 | 12075841 | 7.69   | 381386  |
| 2  | Catechol              | 15.601 | 23238735 | 14.79  | 762836  |
| 3  | Gallic acid           | 5.388  | 31071773 | 19.77  | 1571949 |
| 4  | Quercetin             | ---    | ---      | ---    | ---     |
| 5  | Resorcinol            | 12.353 | 14270535 | 9.08   | 438554  |
| 6  | Tannic acid           | 32.743 | 766953   | 0.49   | 35517   |
| 7  | Vanillin              | 28.998 | 67301243 | 42.83  | 1953291 |
| 8  | Salicylic acid        | ---    | ---      | ---    | ---     |
| 9  | Acetyl Salicylic acid | 39.188 | 3486930  | 2.22   | 110858  |
| 10 | Benzoic acid          | 40.507 | 4155117  | 2.64   | 135628  |
| 11 | Phloroglucinol        | ---    | ---      | ---    | ---     |
| 12 | Ascorbic acid         | 2.794  | 766998   | 0.49   | 149779  |

Collection of samples: The standard phenolic compounds were purchased from reputed manufacturers such as Quercetin Dihydrate & Salicylic Acid from Sisco Research Laboratories Pvt. Ltd, Mumbai; Aspirin, L-Ascorbic Acid, Tannic Acid, Benzoic Acid, Gallic Acid & Catechol from Nice Chemicals Pvt. Ltd, Cochin; and Vanillin, Ellagic Acid, Phloroglucinol GR & Resorcinol Recryst from Merck Specialties Pvt. Ltd, Mumbai.

Preparation of sample standards: 6mg of each standard compound was dissolved in 10 ml of HPLC grade Methanol resulting in a sample concentration of 600 µg/ml. This was sonicated and then passed through Whatman Nylon Membrane Filter (0.45µm & 47mm diameter) before injecting it in the column.

Specification of the HPLC instrument: Analysis of all the standard samples was performed using 515 HPLC pumps and 2489 UV/VIS detectors of Waters company, USA, having reverse phase water guard Column: Symmetry C18 (5µm, 4.6\*250mm) and Hamilton microliter syringe using an injection volume of 20 µl. The data analysis was done using Empower 2 software.

Methods for HPLC analysis: Four HPLC methods using different mobile phases selected on the basis of varying gradations of solvent systems in specific retention times and elute detections were used<sup>[8]</sup>. In general, it was found

that UV detection was quite pronounced at wavelengths of 280 nm and 360 nm using a flow rate of 1.0 ml/min. The mobile phase gradient elutions and run times used in the four methods were as follows:

#### Method A

- 1.Total run time was 22 minutes
- 2.Gradient elution of two solvents was used- Solvent A (Methanol) and Solvent B (Acetic acid in water (1:25))<sup>[13]</sup>. The gradient program was begun with 100 % B and was held at this concentration for the first 4 minutes. This was followed by 50 % eluent A for the next 6 minutes after which concentration of A was increased to 80 % for the next 10 minutes and then reduced to 50 % again for the following 2 minutes.

#### Method B

1. Total run time was 25 minutes
- 2.Gradient elution of two solvents was used- Solvent A (Methanol) and Solvent B (1% Acetic acid in water)<sup>[13]</sup>. The gradient program was started with 60 % of A and then changed to obtain 35, 10, 60 and 60 % of eluent A at 5, 10, 15 and 20 minutes respectively.

#### Method C

- 1.Total run time was 30 minutes
- 2.Gradient elution of two solvents was used- Solvent A (Acetonitrile) and Solvent B (0.1% Phosphoric acid in

water)<sup>[14]</sup>. The gradient program was begun with 85 % B and was held at this concentration for the first 12 minutes. This was followed by 75 % eluent B for the next 10 minutes after which its concentration was again increased to 85 % for the next 8 minutes.

#### Method D

1.Total run time was 45 minutes

2.Gradient elution of two solvents was used- Solvent A (Acetonitrile) and Solvent B (0.1% Phosphoric acid in water)<sup>[15]</sup>. The gradient program was started with 8 % of A for the first 35 minutes and then increased to 22 % for the next 10 minutes before bringing it down again to 8 %.

## RESULTS AND DISCUSSION

Method A: Chromatograms of ten out of twelve standard phenolic compounds namely Ellagic acid (8.9 min), Catechol (7.912 min), Gallic acid (6.85min), Quercetin (10.34 min), Resorcinol (7.64 min), Tannic acid (4.10 min), Vanillin (8.80 min), Salicylic acid (10.92 min), Acetylc Salicylic Acid (9.90 min) and Benzoic acid (9.90 min) which were clearly eluted at different retention times could be obtained using the mobile gradient phase consisting of methanol and acetic acid in water (1:25) for 22 minutes run time when each of these compounds were analyzed individually. Phloroglucinol and Ascorbic acid were not separated following the same method. The HPLC profile of all these 12 phenolic compounds when taken together showed the same sequence of elution using this method, with the combined Chromatogram giving the appearance of being a collage of the nine individual chromatography profiles, the elution times of individual peaks being quite close to the above-mentioned times (Figure 1 and Table 1).

Method B : HPLC profiles of eleven out of twelve standard phenolic compounds namely Ellagic acid (4.40min), Catechol (3.60 min), Gallic acid (2.67min), Quercetin (18.76 min), Resorcinol (3.10 min), Tannic acid (2.47min), Vanillin (3.84 min), Salicylic acid (19.51 min), Acetylc Salicylic Acid (4.73 min), Benzoic acid (6.75 min) and Phloroglucinol (2.60 min) having different elution times could be obtained when each compound was analyzed individually using the mobile gradient phase consisting of methanol and 1% acetic acid in water during 25 minutes run time. However, Ascorbic acid was not separated during this method. The Chromatographic finger print of these 12 phenolic compounds when mixed together exhibited the same elution sequence; the combined Chromatogram resembling a synchronized collation of the individual HPLC profiles, the individual peak times being quite similar to the above-mentioned times (Figure 2 and Table 2).

Method C: Chromatograms of eight phenolic compounds, i.e., Ellagic acid (11.86 min), Catechol (9.08 min), Gallic acid (3.50 min), Resorcinol (7.15 min), Vanillin (12.77 min), Acetylc Salicylic Acid (17.46 min), Benzoic acid (19.19 min) and Ascorbic acid (2.56 min) having retention times mentioned above could be obtained using mobile gradient phase of Acetonitrile and 0.1% Phosphoric acid in water at 30 minutes run time when each chemical was individually analyzed. Quercetin,

Tannic acid, Salicylic acid and Phloroglucinol were not separated in this method. The HPLC finger prints of a mixture consisting of these 12 phenolic compounds revealed the same pattern of elution using this method, with the combined Chromatogram appearing like an aggregation of individual profiles (Figure 3 and Table 3). Method D: Using this method involving a mobile gradient phase consisting of Acetonitrile and 0.1% Phosphoric acid in water for 45 minutes run time, the HPLC analysis of 9 phenolic compounds, namely Ellagic acid (31.88 min), Catechol (15.60 min), Gallic acid (5.38 min), Resorcinol (12.35 min), Tannic acid (32.74 min), Vanillin (28.99 min), Acetylc Salicylic Acid (39.18 min), Benzoic acid (40.50 min) and Ascorbic acid(2.79) could be done while Quercetin, Salicylic acid and Phloroglucinol were not separated. The HPLC profile of these 12 phenolic compounds when combined together exhibited the same sequence of elution, with the combined Chromatogram of nine phenolic compounds giving the appearance of being a superimposition of individual chromatography profiles with similar peak elution times (Figure 4 and Table 4).

## DISCUSSION

The HPLC fingerprints of these standard phenolic compounds obtained using the methods described above would serve the purpose of established benchmarks for future plant research. The qualitative and quantitative analysis of the actual phenolic compounds present in any unknown plant sample would be facilitated by means of comparison with such standard chromatograms, enabling identification & confirmation of presence of any of these 12 common phenolic compounds in the research sample. The use of multiple methods involving different mobile gradient phases would increase the validity and reliability of the obtained results.

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