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Mini-Review of Analytical Methods used in Quantification of Ellagic Acid

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Abstract: Ellagic acid is an important phytochemical present in different plants such as Strawberry, Grapes, Blackberry, Raspberry etc. Chemically, ellagic acid is 2, 3, 7, 8-tetrahydroxychromeno [5, 4, 3-cde] chromene-5, 10-dione. It is an organic heterotetracyclic compound resulting from the dimerization of gallic acid molecules by oxidative aromatic coupling with intramolecular lactonization. Ellagic acid has been reported for various pharmacological activities such as anti-inflammatory, neuroprotective, cardioprotective, antioxidant, anti-mutagenic etc. Various analytical methods based on spectrophotometry, chromatography, hyphenated techniques, capillary zone electrophoresis etc. have been developed for identification and quantification of ellagic acid in natural sources and formulations. The present review provides detailed information on quantitative analysis of ellagic acid present in Strawberry, Grapes, Blackberry, Raspberry, Cranberry; *Syzygium cumini* seed extract, *Woodfordia fruticosa* plant extract, *Potentilla species* extracts etc. It also focuses on analytical methods for quantification of ellagic acid in herbal and traditional formulations such as Ashwagandharishta, Triphala churna, Dhatrinisha churna, Arjunarishta, Manjisthadi churna.

Key Words: Ellagic acid, polyphenols, analytical methods, extracts, formulations, HPLC, HPTLC

1 Introduction

Tannins are phenolic compounds found in different plants. They precipitate proteins and also form complex with minerals, starch and cellulose. Tannins are usually divided into two groups: hydrolysable tannins and condensed tannins. Hydrolysable tannins include gallotannins and ellagitannins. These are usually present in small amounts in plants. These phytoconstituents are environmentally very important because they are easily water soluble at most pH's and they tend to combine with toxic metal ions which may reduces bioavailability.

Depending upon nature of phenolic acid, hydrolysable tannins are divided into two subclasses namely gallotannins and ellagitannins [3]. Gallotannins show presence of gallic acid whereas ellagitannins show presence of ellagic acid [4]. Upon hydrolysis of gallotannins yields sugar and gallic acid, while ellagitannins contain hexahydroxydiphenic acids that produce ellagic acid after hydrolysis [5,6].

Different pharmacological activities of ellagic acid have been reported such as antioxidant [7,8,9], antimalarial [10], anti-inflammatory [11], antihepatotoxic [12], antiproliferative [9,13,14], anticholestatic [15,16], antifibrogenic [12], antihepatocarcinogenic [18,21] and antiviral [20–22]. Ellagic acid has also been reported for its potential neuroprotective action [23]. It has also shown significant effects in management of metabolic syndrome [24]. Ellagic acid is having cytotoxic, antitumor, and anticancer effects [25]. It has also been reported for its cardioprotective effects [26,27] and used as an anti tubercular agent [28]. Ellagic acid also improves the hepatic architectural and functions against toxic and pathological conditions [15]. Derivatives of ellagic acid like urolithins and 4,4' -Di -*O*-methyl ellagic acid have been reported to inhibit colon cancer cell proliferation [29]. Moreover, ellagic acid has been reported to block activated pancreatic stellate cells [30]. Ellagic acid also has apoptosis inducing activities [31]. UV radiation causes oxidative stress through production of ROS, which disrupt the endogenous antioxidative system of the skin cells and may lead to skin inflammatory disorders, depigmentation, photo aging, and carcinoma. Ellagic acid has shown protective effects in these conditions [32,33]. The intake of ellagic acid varies in humans around the world and depends on both the region and the life-style. It can usually be obtained directly in its free form or as ellagitannins, which are hydrolyzed by the enzyme ellagitannase (ellagitannin acyl hydrolase) to release

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ellagic acid and other relevant metabolites [34]. Biochemical and physiological actions of ellagic acid includes inhibition of glutathione S-transferase, contact activation in blood coagulation. It is also used for the assay of factor XIIa in plasma [35]. Ellagic acid is present in Strawberry [2], Grapes [36], Blackberry [1], Raspberry [2], Cranberry [37], Pomegranate [38,39], Guava [40], Walnuts [41,39] and Blueberry [2].

To know various physicochemical properties of natural products different analytical instruments are used. The very basic and important is UV Vis Spectrophotometer to measure the maximum absorbance of the compound. UV-visible spectroscopy (radiations with wavelengths between 10 and 1000 nm) offers information about the transition of the most external electrons of the atoms. UV-visible spectroscopy can be used to determine many physicochemical characteristics of compounds and thus can provide information to identify a particular compound [42].

One of the important biophysical techniques that enables us the separation, identification and purification of the phytoconstituent is chromatography. It is used for quantitative and qualitative analysis. It separates a mixture of phytoconstituents into their different components on the basis of their molecular structure and molecular composition involving a stationary phase and a mobile phase [42]. Herbal medicinal products and dietary supplements and now it become part of life that people take to improve their health. It is very essential to develop authentic analytical methods for these supplements which can reliably estimate the phyto-chemical composition, including quantitative analyses of bioactive compounds and other major phytoconstituents [43].

High-performance thin layer chromatography (HPTLC) is one of the sophisticated instrumental techniques for qualitative and quantitative analysis of the herbs and herbal drugs. HPTLC has a highly sensitive scanning densitometry for the rapid identification and quantification of phytoconstituents [46]. The HPTLC fingerprint is also suitable for rapid and simple authentication and comparison of the subtle differences among samples with identical plant sources but different geographic locations and hence is a very important tool in the herbal drug industry [43,44].

To investigate large numbers of plant secondary metabolites, it is important to have some strong rugged and robust technique which can analyze a number of samples without deviation from the results. All of these things can be achieved correctly by High-Performance Liquid Chromatography (HPLC) analysis, which is an extremely versatile technique [45]. The reversed-phase method is capable to handle compounds of a different molecular mass and having diverse polarity. RP-HPLC has got analytical and preparative application in the area of purification and separation of phytoconstituents [46,47].

Hyphenated techniques also have a unique application in the natural product analysis [48]. Current advancements in hyphenated analytical techniques have remarkably widened applications of LC–MS, GC–MS, LC–NMR, LC–PDA, LC–FTIR, LC–NMR–MS and CE–MS to the analysis of complex biomaterials, especially natural products [48]. Considering all the facts and facets, this review article mainly focuses on the different analytical techniques or methods available for quantification of the ellagic acid.

2 Chemistry, Properties and biogenesis

Ellagic acid belongs to the class of hydrolysable tannins. Its molecular weight is 302.194 g/mol and molecular formula is $C_{14}H_6O_8$. The reported melting point of ellagic acid is 350°C [20]. It is highly thermostable due to presence of four rings in the structure which represent lipophilic dominance, and the four phenolic groups and the two lactones representing the hydrophilic zone [49] (Figure 1). Ellagic acid is an organic heterotetracyclic compound resulting from the dimerization of Gallic acid by oxidative aromatic coupling with intramolecular lactonisation of both carboxylic acid groups of the resulting biaryl. It is a cyclic ketone, also a lactone, also a member of catechols and a polyphenol [50]. Ellagic acid appears as cream-colored needles (from Pyridine) or yellow powder, odorless powder. It is slightly soluble in alcohol or water, soluble in alkalies, in pyridine. It is practically insoluble in ether. When heated to decomposition it emits acrid smoke and irritating vapors [51]. Ellagitannins are found naturally in plants and vegetables as hexahydroxydiphenoyl-glucose esters, ingestion of these results in hydrolysis which releases ellagic acid which is weakly absorbed in the small intestine and stomach [29].

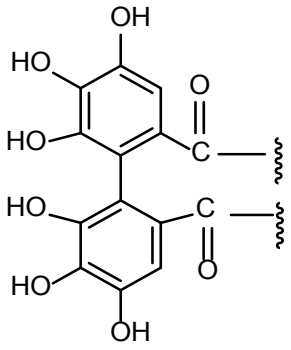

Figure 1 Structure of Hexahydroxidiphenic Group of Ellagitanins

Plants phenols are mostly produced from the phenylpropanoid and shikimate pathway [52]. Ellagic acid is a polyphenolic compound obtained commercially by chemical extraction of plant materials mentioned in the source with the mixture of acid-methanol as solvents and hydrolysis with concentrated HCl or H_2SO_4 [12,20].

Ellagitannins are produced from gallotannins by oxidative interaction with minimum two galoyl units, which originates from hexahydroxydiphenic acid (HHDP). After hydrolysis of ellagitannins, this group will release and impetuously rearranged or lactonised to form the ellagic acid [53] (Figure 2). Aguilera-Carbo and co-workers in 2007 reported production of ellagic acid by fungal solid state culture using polyurethane foam (PUF) as support from an aqueous extract obtained from pomegranate husk (*Punica granatum*) [54]. It is combustible and incompatible with strong oxidizing agents.

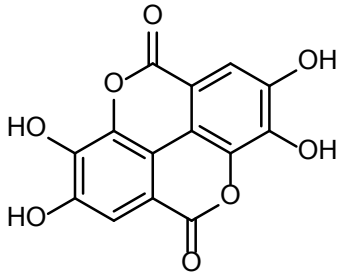

Figure 2 Structure of Ellagic acid

3 Analytical Methods

Analytical studies of herbal drugs and formulations are essential to get quantitative data for the presence of active constituents. To obtain the analytical data various instrumental techniques like spectroscopy, chromatography, electrophoresis, thermal methods, chemiluminescence and hyphenated techniques are widely used. Out of all these techniques, a few of the most versatile techniques are discussed for quantification of ellagic acid from different natural sources and formulations.

3.1 Spectrophotometry

Ultra Violet Spectrophotometry is a method used to measure absorbance of light by measuring the intensity of light passing through the chemical substance. Scientists from different backgrounds use this method as a basic and starting point of research. This data gives a basic idea about solubility and absorption profile of chemical.

Ellagic acid offers maximum absorption of (ethanol) 366, 255 nm with log ε 3.93, 4.60 (Pubchem, 2019). Considering the complex nature and structure of ellagic acid, Chen Jia-hong and co-researchers have made an attempt to dissolve the ellagic acid in dilute NaOH solution, as it wasn't dissolving in any of the organic solvents and in water. They have reported UV characteristic absorption peak of ellagic acid at 357 nm [55]. Budavari and co-researchers have reported 357 nm as a maximum absorption peak in UV region. They also reported that, ellagic acid is sparingly soluble in alcohol and insoluble in water [56]. Bala and co-investigators have determined the solubility of ellagic acid in water and methanol by using the validated UV spectroscopic method and they have reported maximum absorption at 255 nm and 360 nm [49] (Table 1). Maximum absorptions of ellagic acid have been reported at 255 nm, 254 nm and 360-368 nm in UV spectroscopic analyses [57,58].

Ellagic acid is a weakly acidic drug which shows an increase in solubility with pH and having reported p*K*a value of 5.6 [59]. It has four phenolic groups which are weak in acidic nature and should exhibits four dissociation constants [60,61]. Below a pH 5.6, ellagic acid exists in a mono-deprotonated form whereas, at a pH higher than 5.6, deprotonation occurs at the two hydroxyl group positions [59]. Also the report showed that, solubility of ellagic acid increases after it has lost two hydrogen's per molecule. Attempts to increase the solubility of EA by increasing the pH resulted in development of dark greenish brown color, which could be due to the hydrolysis of lactone moieties (pH 9.6, 10^{-5}M NaOH) [59,49].

In IR Spectroscopy, ellagic acid presents specific regions of absorption. Analysis of total polyphenols by IR spectrum was done by Goriparti and fellow researcher after

Table 1 Lambda Max of ellagic acid with different solvent systems by UV Spectrophotometry

Compound	Solubility	Lambda Max	Reference
Ellagic Acid	Ethanol	366 nm	[50]
Ellagic Acid	Dil. NaOH	357 nm	[55]
Ellagic Acid	Methanol	255 nm and 360 nm	[49]
Ellagic Acid	100% methanol	255 nm	[57]
Ellagic Acid	70% acetone in water (v/v)	254 and 360-368 nm	[58]

scanning between 4000 to 10,000 cm^{-1} they have reported, the IR spectrum of the compound exhibits -OH broad band's stretching in the range, 2800-3700 cm^{-1}, also at 1725 cm^{-1} C=O stretching. An aromatic ring vibration band was observed in between, 1669-1500 cm^{-1}. The one which is at 1190 and 1052 cm^{-1} are due to ester linkage. Another signal at 751 cm^{-1} has been assigned to aromatic C-H bending vibration [62].

3.2 Chromatography

Various chromatographic methods have been reported for the identification/quantification of ellagic acid in extracts and in formulations.

3.2.1 High Pressure Thin Layer Chromatography (HPTLC)

High Performance Thin Layer Chromatography is a sophisticated and automated form of thin-layer chromatography [44], along with better and higher detection and separation capability [63]. The main application of this technique in the analysis of phytochemicals [64], quantification of phytochemicals [65], fingerprinting of formulations [44] and also it has wide role in checking the quality of formulations [66].

3.2.1.1 HPTLC Analysis of Extracts

Syzygium cumini seed extract was studied by Dalavi and group to analyze effect of ellagic acid for antibacterial activity. They have performed HPTLC study to determine the effect of accelerated storage on extract and markers of plant on the antibacterial activity after a six month study. The peak for ellagic acid was observed at R_f of 0.47±0.02. The results suggest that, the reduction in the antibacterial potential of the extract along with the concomitant reduction in the percentage assay over a six month study [67]. The details related to the parameters of the method are mentioned in the table 2.

Table 2 Quantitative analysis of ellagic acid by HPTLC methods

Sample	Matrix	extraction element	Chromatographic conditions	Detection	RT (in Min)	Reference
HPTLC Analysis of Extract:						
Syzygium cumini seed	Extraction	Ethanol	toluene: ethyl acetate: formic acid (6:6:1.5v/v/v)	271 nm	0.47 ±0.02	[67]
Abrus precatorius, *Phyllanthus maderaspatensis, Nymphaea alba* Linn	Extraction	Methanol	Toluene: ethyl acetate: formic acid: methanol (3:3:0.8:0.2 v/v/v)	280 nm	0.46	[68]
Woodfordia fruticosa	reflux	HPLC water	toluene: chloroform: ethyl acetate: formic acid (2:6:6:2)	254 nm, 366 nm	-	[69]
Potentilla species	extraction	Methanol	Toluene: ethyl formate: formic acid (6:4:1 v/v/v)	254 nm	-	[70]
Rosa hybrida	Extraction	-	CAN:H_2O:HCOOH (50:50:5)	270 nm	-	[71]
HPTLC Analysis of Ayurvedic / Traditional formulation:						
Ashwagandharishta	decoction	Methanol	Toluene: ethyl acetate: formic acid: methanol (9:9:3:0.6 v/v/v/v)	285 nm	0.46 ± 0.02	[72]
Triphala churnam	Extraction	Methanol	Toluene: Ethyl Acetate: Formic Acid: Methanol (3:3:0.8:0.2 v/v)	280 nm	0.47	[73]
Dhatrinisha churna	Extraction	methanol	toluene : ethyl acetate : methanol : formic acid (16: 14: 1: 4 v/v)	330 nm	-	[74]
Arjunarishta	Decoction	methanol	Toluene: ethyl acetate: formic acid: methanol 9:9:3:0.6 (v/v)	285 nm	0.46	[75]
Manjisthadi churna	Extraction	Methanol	toluene: ethyl acetate: methanol: formic acid (10:9:6:5 v/v)	280 nm	0.72	[76]

Ellagic acid content was determined by HPTLC in flowers of *Nymphaea alba* Linn, whole plant of *Phyllanthus maderaspatensis* Linn, and seeds of *Abrus precatorius* Linn. Extracts were prepared by Soxhlet extraction and dilutions were made in prepared methanol. Researchers have reported simultaneous instrumental precision for gallic acid and ellagic acid was 0.083 and 0.78, repeatability 1.07 and 1.50 (% CV). They have also reported accuracy of the simultaneous method by a recovery study at two different levels and the average percentage recovery was found to be 101.02% for gallic acid and 102.42% for ellagic acid [68] in whole plant.

The content of ellagic acid was determined in *Woodfordia fruticosa* plant which was procured from three different states of India namely Himachal Pradesh, Telangana and Karnataka. The extract was prepared by refluxing sample with 50 ml of 70% ethanol for half hour. The report showed that the presence of ellagic acid in the extracts gave bands at R_f value of 0.30 at UV 254 and 366 nm, which was in concordance with standard ellagic acid [69].

Bazylko and coresearcher performed fingerprinting analysis of eleven *Species of Potentilla L. (Rosaceae),* which were collected during different time and extracted with methanol. Their study aimed for HPTLC fingerprinting methods for different polyphenolic compounds including ellagic acid in selected *Potentilla species*. A mobile phase of toluene: ethyl formate: formic acid (6:4:1 v/v/v) was used on HPTLC silica gel 60 F_{254} as stationary phase and developed the method. They have reported the presence of ellagic acid at R_f 0.13 for all selected eleven species [70].

Polyphenol content of the various parts of plant *Rosa hybrid* was determined by HPTLC. Sixty compounds were reported in different parts of plant *Rosa hybrid* like wood, shoots, early buds, buds before flowering, flowers and leaves, including hydrolysable tannins mainly ellagic acid [71].

3.2.1.2 HPTLC Analysis of Ayurvedic / Traditional Formulations

Tiwari and co-researchers performed HPTLC on the Ashwagandharishta which is a Traditional Ayurvedic formulation containing roots of Ashwagandha, which contains ellagic acid. Studies were performed on three samples, viz, samples prepared by a traditional method, samples prepared by a modern method and samples procured from a local market. A method was developed for the said preparation, and the percentage of ellagic acid was found to be 0.0191, 0.0189 and 0.0188% w/w respectively [72].

Triphala churna consist of one part each of *Terminalia chebula, Embellica officinalis* and *Terminalia bellirica*. N.S. Jeganathan and K. Kannan developed HPTLC method for quantitative estimation of ellagic acid in the laboratory prepared formulation and the marketed formulation of Triphala churna. Extraction was done using the Soxhlet extraction technique with methanol and ethyl acetate. In the laboratory prepared formulation at 280 nm, 0.2% and 0.5% of ellagic acid was found in methanolic and ethyl acetate extract respectively. While in the commercial formulation, ellagic acid was obtained in methanolic and ethyl acetate extract (0.05% w/w and 0.4% w/w respectively) [73].

Dhatrinisha churna is an Ayurvedic formulation used for the treatment of hyperlipidaemia. Patel and co-researchers had estimated the ellagic acid content by HPTLC method and also validated the method as per International Conference on Harmonization (ICH). Results showed r^2>0.997 for linear correlation coefficient, the percent RSD was less than 1.92% and recoveries were between 96.60-101.4% [74] (Table 2).

An Ayurvedic formulation called Arjunarishta, also known as Parthadhyarishta, is being used for treatment in cardiovascular disorders. A HPTLC method was developed for ellagic acid and amount of ellagic acid in Arjunarishta-T (prepared by traditional method) and Arjunarishta-M (prepared by modern method) and also in its marketed formulation was found to be 0.0361, 0.0360 and 0.0359% w/w respectively. Recovery was found between 99.82-99.93% in Arjunarishta-T, 99.89-100.23% in Arjunarishta-M and 100.04-100.13% in the marketed formulation [75] (Table 2).

In the traditional Ayurvedic system of medicine, hyperlipidaemia is treated by Manjisthadi churna. The HPTLC method of methanol extraction of churna was developed using toluene: ethyl acetate: methanol: formic acid (10:9:6:5 v/v) as a mobile phase. The extract was used for HPTLC on silica gel plates. The R_f of standard ellagic acid was found to be 0.72 with densitometric scanning at 280 nm of the marker. The ellagic acid content was 0.534% w/w in *Manjisthadi churna*. Recovery values from 100.75-102.33% showed the reliability and reproducibility of the method [76] (Table 2).

3.2.2 High Pressure Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) is a technique used to separate analytes from different samples. It depends upon the chemical structure, molecular weight and nature of the analytes [77].

HPLC can be used for analysis of phytoconstituents, their degradation products and possible derivatives. It can analyze very low concentration of analytes in the presence of co-eluting components. Currently, HPLC offers the added

advantage in the analysis of poly phenolic plant derived and biological matrices like a wide range of commercially available columns with fit-for-purpose properties having new generation sorbents and attachment of two or more columns in a switching mode. Considering all the above unique properties, HPLC is considered as one of the standard methods for analysis of ellagic acid [78].

3.2.2.1 HPLC Analysis of Extracts:

A validated rapid HPLC-PDA method was developed for quantification and identification of ellagic acid in the extracts prepared from the bark and fruits of *Terminalia chebula, Terminalia bellirica, Terminalia arjuna* and *Terminalia catappa*. Authors have reported a limit of detection and a limit of quantification for ellagic acid as 0.5 mg/mL and 1.0 mg/mL respectively. This method is suitable for quantitative analysis as well as quality control of extracts and herbal formulations from all four *Terminalia* species [79]. The details related to the parameters of the method are mentioned in the table 3.

A HPLC method has been developed for estimation of ellagic acid from *Epilobium angustifolium* (*Canadian willow* herb) extract. Authors have reported correlation

Table 3 Quantitative analysis of ellagic acid by HPLC methods

Sample	Matrix	Sample preparation	Column	Chromatographic conditions	Detection	RT (in Min)	Reference
HPLC Analysis of Extracts							
Terminalia chebula, Terminalia bellirica, Terminalia arjuna and Terminalia catappa	Extracted with hydro alcoholic solvent (3 : 7, water : methanol)	500 mg/ mL, HPLC grade methanol	RP-18 column (SunFire, Waters, USA) with 5 µm, 4.6 mm id,150 mm length	acetonitrile (A) And (0.05%) TFA in water (B, pH 2.25)	PDA	-	[79]
Epilobium angustifolium	--	Methanol (HPLC grade)	Sunfire C18 column, 5µm (4.6 × 250 mm)	A-0.1% Orthophosphoric acid solution and B- Acetonitrile	UV	16	[80]
Eugenia uniflora	Extraction	Ethanol	Supelco Analytical C18 column (250 × 4.6 mm, 5 µm)	Water: acetonitrile (85:15)	UV	12.22	[81]
Woodfordia fruticosa	Reflux	HPLC grade water	C18 (250 mm × 4.6 mm), 5 µm	acetonitrile: 5 mM potassium dihydrogen orthophosphate (KH_2PO_4) (95:5 v/v) buffer pH 2.5 with dilute orthophosphoric acid	UV	12.3	[69]
Punica granatum (Pomegranate Rinds)	Extraction	50% methanol	Zobax SBC18 column (250 × 4.6 mm id; 5 µm)	Methanol: ethyl acetate: potassium dihydrogen phosphate: phosphoric acid(both at 0.05 M) in the ratio 34:2:64 (v/v/v)	DAD (G1365B-MWD)	10.32	[38]
Geum rivale	Extraction	diethyl ether	Lichro CART (250 × 4 mm)	A: (0.5% water solution of H_3PO_4) and B: ACN	UV/VIS	-	[82]
Pomegranate husk	Extraction	60% ethanol in water	RP BDS Hypersil C18 (250 × 4.6 mm id, 5 µm)	90% methanol and 10% H_2O	-	13.19	[62]

Jatropha dioica, Euphorbia antisyphyllitica, Turnera diffusa, Flourensia cernua, Punica granatum	Extraction	EA in methanol	Optisil ODS column (5 µm, 250 × 4.6 mm)	Acetonitrile (A) and water : phosphoric acid (pH 2.5, B), 30 : 70 v/v	PAD	5.1	[83]
Strawberry	Extraction, 80% methanol in water v/v and 70% acetone in water v/v	-	Betasil C18 column (250 mm × 2.1 mm id, 5 µm) 5 µm C18 guard column (4.0 mm × 2.1 mm id)	(A) Acetic acid: water (2:98, v/v) and (B) acetic acid: acetonitrile: water (2:50:48, v/v/v).	DAD	-	[58]
Myrtle berries *(Myrtus communis)*	Extraction	Alcoholwa-ter mixture	C18 (250 mm × 4.6 mm, 5 µm, Waters) column	A, 0.2 M phosphoric acid, and mobile phase B, acetonitrile 0.2 M phosphoric acid (80:20, v/v)	HPLC-UV	-	[101]
Rosa hybrida	Microwave Assisted Extraction	-	C18 Nucleodur sphinx ,150 × 4.6 mm, 5 µm	A: 0.1% formic acid in water, B: 0.1% formic acid in methanol	DAD	-	[71]
Myricaria bracteata leaves	Extraction	ethanol: water mixture (70:30, v/v)	Zorbax SB-C18 Column (4.6 mm × 150 mm, 5 µm id)	MeOH (A) and 0.1% orthophosphoric acid in water (B)	DAD	-	[84]
Phyllanthus emblica, P. fraternus, P. amarus and P. niruri	Extraction	ethanol	ThermoBetasil C8 column (250 mm×4.5 mm, 5 µm)	0.1% formic acid in water (A) and 0.1% formic acid in methanol (B)	DAD	-	[99]
Muscadine grapes	Extraction	acidified methanol	250 mm × 4.6 mm Acclaim 120 C18 column (Dionex, Sunnyvale, CA) C18 guard column	water (phase A) and 60% (v/v) methanol (phase B) both adjusted to pH 2.4 with orthophosphoric acid	PDA-100 detector	-	[98]
HPLC Analysis of Herbal Product:							
Roots of Salacia species, leaves of *Lagestroemia parviflora* and fruit rind of *Garcinia indica*	Extraction	methanol	Phenomenex C18 column (250mm × 4.6mm id, 5µm pore size)	A: acetonitrile B: buffer solution (0.03% v/v Phosphoric acid) 45:55 v/v	SPD-M20A - PDA	6.09	[90]
Strawberry jam, Blueberry jam and Raspberry jam	Decoction	homoge-nized in methanol	L-column ODS (5 µm, 250×4.6 mm id)	5 mM potassium dihydrogen phosphate solution (pH 2.5)-acetonitrile (41:9 v/v)	DAD	-	[2]
Muscadine grapes	(acetone: H_2O: acetic acid, 70:29.7:0.3, v/v)	-	Zorbax Stablebond Analytical SB-C18 column (4.6 mm ×250 mm, 5 µm	A (0.5% formic acid aqueous solution) and mobile phase B (methanol)	DAD	-	[36]

HPLC analysis of Standard Ellagic acid							
Ellagic acid	Dilution	Stock solution in methanol and working standards with pH 7.4 phosphate buffer	polar reverse phase PEG bonded column	5mM potassium dihydrogen orthophosphate pH 2.5 and acetonitrile (20:80, v/v)	UV (SPD-10Avp)	4.0	[49]
Ellagic acid	Dissolution	Dissolved in 100 % methanol	RP C18 Acclaim, 3 µm, 4.6 ×150 mm column	(A) 0.1 vol. % formic acid in water and (B) 0.1 vol. % formic acid in acetonitrile	PDA (SPD-M10Avp)	-	[57]

coefficient (r^2) >0.999, the % RSD of peak area of standard solution less than 2.0%. The accuracy of the method was checked by the average % recovery and was found within the acceptable range of 98-102% [80].

The quantification of ellagic acid in ethanolic extracts of *Eugenia uniflora* L. (Myrtaceae) leaves was done by the HPLC-UV method and the same was later developed and validated. To study the effects of the ethanol concentration, extraction time and temperature on the ellagic acid, the ultrasound-assisted extraction (UAE) was performed by a Box Behnken design (3^3) combined with response surface methodology. Assuncao and co-researchers have reported that, by the use of UAE, extraction yield of the ellagic acid was highest using ethanol as a solvent [81].

Indian origin plant *Woodfordia fruticosa*, is widely used as folk medicine for the treatment of various diseases. Researchers have collected samples of *Woodfordia fruticosa* from three different states of India viz, Telangana, Karnataka, Himachal Pradesh and performed the HPLC analyses of the extract of flowers of *Woodfordia fruticosa* to determine the presence of ellagic acid. The report showed that, the peak at a retention time of 12.3 min was common in all the three samples and matches with the ellagic acid standard sample [69].

Ellagic acid was analyzed in hydrolyzed extracts of *Punica granatum* (pomegranate rinds) by the HPLC method and the report showed that retention time for ellagic acid was 10.32 min, detection limit 2.8 µg/mL, relative standard deviation for the within-day precision was 1.23%, and average recovery was 98.32%. The report showed that this method can be applied to the quality control of pomegranate rinds [38].

Investigation of biologically active constituent of *Geum rivale* was done by RP-HPLC. Owczarek and co-researchers have analyzed a total of eleven phenolic acids from aerial parts and eight in underground parts of the plant. The extract was prepared with petroleum ether and chloroform using a Soxhlet apparatus. They also reported a retention time of ellagic acid around 40 min [82].

Finely ground pomegranate husk was extracted three times with 60% ethanol water solution, and then kept for five hours in 5.0% H_2SO_4 for acid hydrolysis. After that, recrystalization was done with methanol to obtain over 90% pure ellagic acid. Later on it was recrystallized from hot pyridine and resulted in >99% pure crystalline ellagic acid. This ellagic acid was then used for HPLC analysis, which resulted in a RT of 13.79 min [62].

Five different plants, *Jatropha dioica* branches, *Euphorbia antisyphyllitica* branches, *Turnera diffusa* leaves, *Flourensia cernua* leaves and *Punica granatum* husk were collected, finely powdered and subjected to ellagic acid extraction along with HPLC analysis. The report showed that, different quantities of ellagic acid in these plants like, *Punica granatum* turning 33.79±7.43, *Punica granatum* red 12.80±5.83, *Euphorbia antisyphyllitica* 2.18±0.39, *Flourensia cernua* 1.59±0.96, *Tumera diffusa* 0.87±0.59, *Jatropha dioica* 0.81±0.43 mg/g [83].

HPLC analysis of *Fragaria × ananassa* was done by Aaby and co-researchers using two mobile phases acetic acid: water (2:98 v/v) and acetic acid: acetonitrile: water (2:50:48 v/v/v) with a Betasil C18 column. Aaby and co-researchers have reported a retention time of 55.1 min for ellagic acid at 368 nm [58].

HPLC-DAD analysis of six different parts of the plant *Rosa hybrid* was carried out for the initial identification of the polar compounds present in each six different organs (like wood, shoots, early buds, buds before flowering, flowers, leaves). Using a detection wavelength of 270 nm, Riffault and co-researchers have reported 29.2 min retention time for ellagic acid [71].

Myricaria bracteata leaves showed a retention time of ellagic acid at 10.6 min. The mobile phase consisted of methanol and 0.1% orthophosphoric acid in water 50:50 (v/v) [84].

The grape seed extracts were analyzed by HPLC-DAD system and the results showed the presence of ellagic acid in the processed methanolic and acidified methanolic extract. The reported concentration of ellagic acid in the grape seed extract was 0.08 mg/g with retention time of 22.7 min and regression (R^2) was 0.9975 [85].

The juice of *Syzygium cumini* fruits and dehydrated powders obtained by foam mat drying and lyophilization of juice were analyzed for presence of phenolic compounds. HPLC with a diode array detection coupled with an electrospray ionization mass spectrometry was used for the analysis. Juice production resulted in the extraction of hydrolysable tannins [86,87,88]. Chromatographic conditions were as follows- Merck LiChrospher RP18e (5 µm) column (25 cm x 4 mm id) with the following elution conditions: linear gradient from 0 to 90% solvent B; solvent A: H_2O:H_3PO_4 (990:1); solvent B: MeOH-H_3PO_4 (990:1); gradient duration: 30 rain; flow speed: 1 ml/min. Detection: UV 280 nm. The reported retention time was 25.0 min for ellagic acid [89].

3.2.2.2 HPLC Analysis of Herbal Formulations

A herbal formulation containing of leaves of *Lagestroemia parviflora*, roots of *Salacia oblonga* and *Salacia roxbhurghii* and fruit rind of *Garcinia indica* was studied for ellagic acid content. The HPLC report for ellagic acid showed LOD and LOQ values as 4.62µg/mL and 14.01µg/mL, respectively. Repeatability and recovery studies were in between 95-103%, precision of the intra-day and inter day percent Relative Standard Deviation by HPLC measurementswere1.05% and 2.22%, respectively. The reported retention time was 6.09min. The developed and validated method was suitable for routine analysis of herbal formulation [90].

Recoveries of ellagic acid from fresh fruits viz. Strawberry, Pineapple and Raspberry and products viz. Strawberry jam, Blueberry jam and Raspberry jam. The analyses showed recoveries of EA from the samples were found to be 90.1 to 98.3% (SD 0.9 to 5.0%). The reported LOD and LOQ were 0.015mg/g and 0.05mg/g for ellagic acid respectively [2].

Sandhu and co-researchers have used HPLC-DAD to identify the phenolic compounds of Muscadine grapes in the seeds, skin and pulp. In the seeds, skin and pulp the average reported percentage of phenolic compounds were 87.1, 11.3 and 1.6% respectively. The presence of ellagic acid is exclusive and unique in Muscadine grapes, and it is available in different forms like ellagitannins, ellagic acid glycosides and free ellagic acid [36].

3.2.2.3 HPLC Analysis of Standard Ellagic Acid:

Studies on ellagic acid by Bala and co-researchers have reported a correlation coefficient of 0.9982. The reported LOD was 0.1 g/ml and range of accuracy was 98-103% indicating a good agreement between the actual and practical values. Bala and co-researchers have also reported elution of ellagic acid on the stationary phase which is bonded with polyethylene glycol resulted in a narrower peak and reduced tailing in comparison to a regular reverse phase C18 column [49].

The ellagic acid standard was dissolved in methanol and then subjected to more detailed validation by Williams and co-researchers. They selected 0.1 vol. % formic acid in water and 0.1 vol. % formic acid in acetonitrile as a mobile phase. The reported results for the validation showed the recovery values of 100 %. Reported LOD and LOQ for ellagic acid were 0.06µg/mL and 0.17µg/mL, respectively [57].

3.2.3 Gas Chromatography-Mass Spectrometry (GC-MS):

Gas Chromatography is well known and sound analytical technique generally used for the quantification, characterization and identification of volatile compounds. It has powerful separation capacity and very sensitive detection which have made this chromatographic technique a useful tool for the analysis [91]. Despite its several advantages, application of this method is limited to the essentials oils because of the chances of thermal degradation of the compounds [92]. When used in combination with mass spectrometry, this method gives high selectivity and sensitivity [93].

Analysis of *Geum rivale* was done by GC-MS whereby aerial and underground parts were used, this belongs to a particular plant family and subfamily which contains large amounts of tannins (polyphenolic compounds) like ellagic acid. The extract of the same was prepared with petroleum ether and chloroform in the Soxhlet apparatus. An Agilent 6890N gas chromatograph with an Agilent 5973 mass detector was used for the GC-MS analysis. The electron impact at 70 eV was used as ionization potential. Owczarek and co-researchers have reported about 130 mg/1g content of tannins in underground parts of the plant [82].

Tannins in the grape, oak and wine extracts were analyzed by GC-MS. The report showed that; ellagic acid was present in oak and wine extract by GC-MS. The analysis was performed using a GC 6890N/MSD 5973B Agilent instrument. The reported retention time for ellagic acid was 33.84 min [94].

GC-MS analysis for the phytoconstituent of *Salacia chinensis* L. was performed. The analysis was done with 60 M RTX 5MS a nonpolar column. A split less mode technique was used to inject 2 µl samples. Mass spectra was recorded over 35-650 atomic mass unit (AMU) range with electron impact ionization energy 70 eV. GraphPad Prism was used to perform statistical analysis. The author has reported the presence of ellagic acid equivalent per gram dry weight into root, stem, leaf, fruit pulp and seeds by different extraction method [95].

3.2.4 Liquid Chromatography – Mass Chromatography (LC-MS):

Liquid chromatography (LC) separates the components of a sample based on differences in their retention strength or affinity for the stationary phase or mobile phase, and then detects the separated components using various analytical techniques such as UV, fluorescence or electrical conductivity based on their properties.

Mass spectrometry (MS) is known for its highly sensitive detection process that ionizes the sample components using various methods, such as electrospray ionization, chemical ionization, fast atom bombardment, laser desorption etc. and then separates the resulting ions in vacuum based on their mass-to-charge ratios and measures the intensity of each ion.

Therefore, LC-MS systems combine the outstanding separation resolution of liquid chromatography with the outstanding qualitative capabilities of mass spectrometry. The MS creates and detects charged ions. The LC-MS data may be used to provide information about the molecular weight, structure, identity and quantity of specific sample components.

Ethanolic extract of Pomegranate peel was analyzed by LC-MS and the identification of ellagic acid was done by comparison with a reference standard. The report showed that the $[M-H]^-$ at m/z 301 confirms the presence of ellagic acid. The same component in MS-MS produces fragments at m/z 258, 229 and 185 using the electrospray ionization technique [96].

Method development and quantification of ellagic acid derivatives was done in four *Drosera* species viz., *Drosera anglica*, *D. intermedia*, *D. madagascariensis* and *D. rotundifolia*. Along with ellagic acid, several other compounds were detected and identified by LC-MS. *Droserae herba* LC-MS analysis was performed with an UltiMate 3000 RSLC-series system (Dionex, Germering, Germany) coupled to a 3D quadrupole ion-trap mass spectrometer equipped with an orthogonal ESI source. For ellagic acid $[M-H]^-$ showed at 300.9 and main fragments include 283.8, 270.8, 256.8, 228.9, 212.8, 200.8, 184.9 [97]. Lee and other co-researchers have reported ellagic acid in Muscadine Grapes by HPLC-ESI-MS. They have reported base peak (m/z) at 301; $[M-H]^-$, MS^2 (m/z) at 301, 284, 257, 229, 185 for ellagic acid [98].

LC-MS analysis of *Fragaria × ananassa* was done by Aaby and co-researchers. They have allowed LC elute directly into ESI interface without splitting. The nebulizer pressure was at 40 psi, dry gas flow at 10 L/min; dry temperature was at 350°C and capillary voltage at 3.5 kV maintained. They have reported a peak at $[M-H]^-$ at *m/z* 301 and MS^2 fragmentation ions in negative mode at *m/z* 257, 229, and 185, typical fragments of ellagic acid [58].

Identification of ellagic acid in *Myricaria bracteata* leaves was done by Chernonosov and co-researchers. They have reported that molecular ion $[M-H]^-$ at m/z 301 and a fragment ion at m/z 284, 257, 229, 185 is of ellagic acid [84].

Ellagic acid and their derivatives in ethanolic extracts of *Phyllanthus emblica*, *P. fraternus*, *P. amarus* and *P. niruri* were detected using HPLC coupled with quadrupole time-of-flight tandem mass spectrometry (HPLC-ESI-QTOF-MS/MS). The authors had reported retention time of 14.07 min for ellagic acid and $[M-H]^-$= 300.999, whereas fragment ions (Relative intensity %) were 283.9975 (66), 245.0085 (36), 229.0135 (45), 200.0103 (58), 185.0242 (39), 173.0232 for ellagic acid [99].

Muscadine grapes HPLC-ESI-MS analysis was done by Lee and co-researchers for the determination of ellagic acid in the isolate. Presence of ellagic acid was confirmed by its *m/z* 301 $[M-H]^-$ion, yielding characteristic ions at *m/z* 257 and 229 upon dissociation [98].

3.2.5 Other Miscellaneous Methods:

Capillary Zone Electrophoresis (CZE) is also a widely used method to determine the concentration of active constituents from the sample of plant extracts. A diverse group of scientists are working on this technique as an alternative tool for the identification of the acid.

Costa and researchers have reported a method for the determination of ellagic acid by CZE and compared the results obtained with GC-MS in *Eucalyptus globulus* wood. Moreover they have reported that, this is the first application of CZE at industrial streams for the detection of ellagic acid from cellulosic pulp production. The reported results by CZE were 959 to 986 mg/kg where as by GC-MS results were 1232 to 1083 mg/kg for ellagic acid. This showed that, both methods do not give significantly different values for ellagic acid [100].

Zhou and co researchers have performed the Capillary Electrophoresis (CE) of the Pomegranate rinds and compared the results with HPLC. The detection limits for HPLC and CE were 2.8 and 2.2 µg/mL, respectively. Average recoveries were 98.32±1.2% for HPLC and 96.52±2.8% for CE. The reported results state that the CE method required less solvent and gave better column efficiency, whilst the HPLC method provided superior precision [38].

4 Conclusion

Tannins are an important class of phytochemicals which have been reported to have various pharmacological activities. Ellagic acid is an important phytoconstituent belonging to the class of tannins. Considering the importance of the ellagic acid in various ailments, it is also necessary to study different analytical approaches to identify and quantify ellagic acid in natural sources. The reports of all the methods described in the present review area compilation of the research done by various scientists on the estimation of ellagic acid on various instruments, and studies done on different kinds of samples. The data suggest that, the methods can be used for raw materials to final products, to determine the concentration of ellagic acid, which is an important aspect for stability of product or formulation.

5 Conflict of Interest:

The authors declare that they have no conflict of interest.

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