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Research Article

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPLC METHOD FOR DETERMINATION OF ELLAGIC AND GALLIC ACID IN JAMBUL SEEDS (SYZYGIUM CUMINI)

Mrinalini Damle* and Nilam Dalavi

Department of Quality Assurance, AISSMS College of Pharmacy, Kennedy Road, Near R.T.O., Pune-411001, Maharashtra, India.

*Corresponding author's email: mcdamle@rediffmail.com

Abstract

Ellagic and Gallic acid are main phytoconstituents of S.cumini seeds. These are the phenolic compounds. An approach for the stress degradation was successfully applied for the development of stability indicating HPLC method for the determination of Ellagic and Gallic acid. Sample was resolved on a Hypersil C18 (250*4.6 mm particle size 5µ) column. The mobile phase consisted of 1% OPA and ACN and in the ratio of 70:30 *v/v* which was sonicated to degas and delivered at a flow rate of 1ml/min at ambient temperature. The retention time of Ellagic acid and Gallic acid was 3.1±0.05 & 4.1±0.05 minutes. Studies were performed using an HPLC system equipped with a UV detector; the response was monitored at 271nm. The method is specific to Ellagic and Gallic acid; it is able to resolve the peak from ethanolic extract of s.cumini seeds and formulation. The calibration curve was linear over the concentration range of 8-24 µg/ml (r^2=0.997, 0.998 resp). The limit of detection for Ellagic acid and Gallic acid was found to be 0.25µg/ml, 0.15µg/ml resp. and the quantification limit was about 0.75µg/m, 0.49µg/ml. The accuracy of the method was established based on the recovery studies. The markers were subjected to acid, base, neutral hydrolysis, oxidation, thermal degradation and photolysis. The method was successfully validated according to ICH guidelines Q2 (R1).

Keywords: Ellagic acid (EA); Gallic acid (GA); High Performance liquid Chromatography; Stability indicating method.

Introduction

Plants have provided mankind with herbal remedies for many diseases for many centuries and even today. In India, herbal medicines have been the basis of treatment and cure for various diseases in traditional methods practiced such as Ayurveda (Unani and Sidha, 2012]). Ellagic acid (EA) and Gallic acid (GA) (Fig. 1) are found to be the active principles of *S. cumini*. *S. cumini* seed is official in Ayurvedic pharmacopeia which plays a vital role in the Ayurvedic system of medicine (Ministry of Health and Family Welfare, 2007). The various phytoconstituents such as glycosides (jamboline), ellagic acid, gallic acid, tannins, fatty oil, steroids, flavonoids, triterpenes are present in *S. cumini* (Ayyanar and Babu, 2012). It has pharmacological actions like antidiabetic (Alam and Rahman, 2012), anti-inflammatory (Muruganandan and Srinivasans, 2001), antibacterial (Kothari and Seshadri, 2011), antiallergic (Lima and Ramos, 2007) and antioxidant (Banerjee and Narendhirakannan, 2011).

The use of plant extracts and phytochemical, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency (Muniappan, 2011).

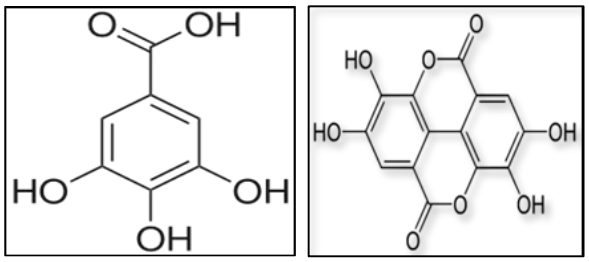

Fig.1: Structure of EA and GA

Literature survey reveals HPLC (Sawant and Prabhakar, 2011; Santos et al., 2013; Syam and Tamizhmani, 2012); Patel and Patel, 2010; Sawant and Prabhakar, 2011; Kardani and Gurav, 2013), HPTLC (Jadhav and Kadam, 2012; Damle and Dalavi, 2015), methods reported for estimation of Ellagic and Gallic acid. But there is no report on validated stability indicating HPLC method for EA and GA acid in *S. cumini.* Hence, a stability indicating HPLC method has been developed in the present work for quantitation of Ellagic and Gallic acid from *S. cumini* seed.

Materials and Method

Chemicals and Reagents

EA and GA purchased from Yucca Enterprises, Mumbai, were used as such, without any further purification. *S.cumini* seeds were purchased from local market & *S.*

cumini seeds were authenticated from Agharker Research Institute, Pune. ACN (HPLC grade), o-phosphoric acid (HPLC grade), were purchased from S. D. fine chemical Laboratories, Mumbai, India, Water (HPLC grade) was obtained using Elga water purification system.

Instruments

Jasco Model V-550 UV-Visible Double beam spectrophotometer, Elga Labwater (PURELAB UHQ-II) HPLC water purification system, Shimdazu Model AY-120 Balance and Jasco HPLC system comprising: Model PU 2080 Plus pump, Rheodyne sample injection port, Hypersil C_{18} Columns, UV detector, Borwin-UV software (version 1.5) was used.

Chromatographic Conditions

The mobile phase consisting 1% o-phosphoric and ACN in the ratio of 70:30 *v/v*, was filtered through 0.45µ membrane filter, sonicated and was pumped from the solvent reservoir. The flow rate of mobile phase was maintained at 1ml/min and the response was monitored at 271 nm with a run time of 10 min. The volume of injection loop was 20µl. The column and the HPLC systems were kept at ambient temperature (Fig. 2).

Preparation of Standard Stock Solution

Standard stock solution of EA and GA were prepared separately by dissolving 10 mg of marker in 10 ml of methanol to get concentration of 1000 µg/ml. From the respective standard stock solution, working standard solution was prepared containing 8-24 µg/ml of EA and GA separately in methanol.

The system suitability parameters are given in Table.1

Stress Degradation Study of Ellagic and Gallic Acid

Stress degradation studies were carried under conditions of acid/ base/ neutral hydrolysis, oxidation, dry heat and photolysis as per ICH Q1A (R2) guideline (1996). For each study, samples were prepared as follows:

1. The blank subjected to stress in the same manner as the marker solution
2. EA, GA working standard solution subjected to stress condition.

Stress condition were optimized terms of strength of reagent and time of exposure to obtain 10-30% degradation.

Alkaline Hydrolysis

To 1.6 ml of 100 µg/ml solution of EA and GA, 1 ml of 0.1 N NaOH (Methanolic) was added. The volume was made up to 10 ml with methanol. The above solution was kept 6hrs at room temperature in dark place. The solution neutralized with hydrochloric acid before injection.

Acidic hydrolysis

To 1.6 ml working standard solution of EA and GA (100 µg/ml) with 1 ml of 0.1N HCl (Methanolic) was added. The volume made up to 10 ml with methanol. Solution was kept for 6 hrs in dark place. The solution neutralized with sodium hydroxide before injection.

Neutral Hydrolysis

To1.6 ml working standard solution of EA and GA (100 µg/ml) with 1 ml water and volume made up to 10 ml with methanol. The solution was kept for 6 hrs in dark place.

Oxidation

To 1.6 ml working standard solution of EA and GA (100 µg/ml) was mixed with 1 ml of 6% v/v solution of H_2O_2 and volume made up to 10 ml with methanol. Solution was kept for 6 hrs in dark place. The solution was warmed on water bath for 10 min cooled & volume was made upto mark.

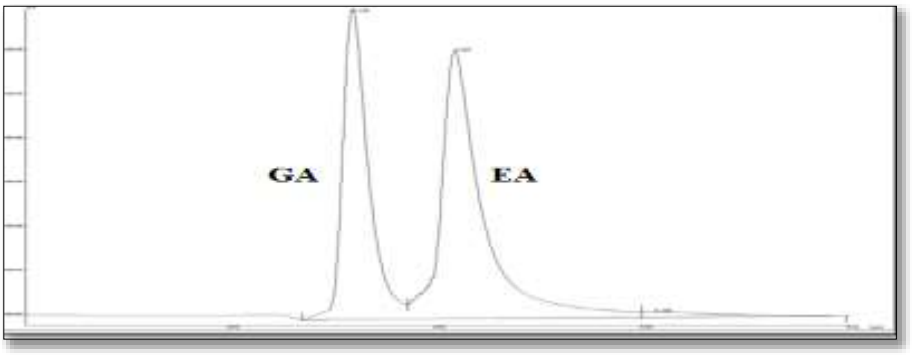

Fig.2: Chromatogram of EA (10 µg/ml) and GA (10 µg/ml)

Table.1: System suitability parameter

Name	RT (Min)	Concentration(µg/ml)	Area	Plates	Asymmetry
EA	4.1± 0.05	16	11834	22814	1.3
GA	3.1±0.02	16	70681	22854	1.5

Degradation under Dry Heat

Dry heat studies were performed by keeping drug sample in oven (60^0 C) for 6 hrs.

Photo-Degradation Studies

Photolytic studies were carried out by exposure of markers to UV light up to 200 watt hours/square meter and subsequently to cool fluorescent light to achieve an illumination of 1.2 million Lux hrs. Sample was weighed, dissolved in methanol and appropriate dilutions were made to get final concentration of 100 µg/, 1.6 ml standard solution of each of EA and GA (100 µg/ml) was mixed in 10 ml volumetric flask and volume was made with mobile phase.

Result and Discussion

Under optimized chromatographic conditions retention Time of EA and GA was found to be 3.1, 4.1±0.05 resp. Degradation was observed for EA and GA during stress conditions like hydrolysis, oxidation, dry heat and photolysis but the peak of degradation product of GA was observed only under acid, alkali hydrolysis. GA showed one degradation product (D1) in alkaline hydrolysis at Rt 8.9, one degradation product (D2) in acid catalysed at Rt 5.1 and one degradation product (D3) in oxidative condition at Rt 3.8. EA does not show any degradation peak under hydrolysis, oxidation, dry heat and photolysis. Summary of stress degradation results is given in Table.2. Peak purity results greater than 980 indicate that EA and GA peaks are homogeneous in all stress conditions tested indicating noninterference of product of degradation. The unaffected assay of EA and GA confirms the stability indicating power of the method (Fig 3).

Table.2: Summary of stress degradation of EA and GA

Stress Degradation Condition	% Recovery		R_t degradtion product (min)	
	EA	GA	EA	GA
Base (0.1 N NaOH, kept for 6hrs)	86.73	81.13	-	8.9
Acid (0.1 N HCl, kept for 6 hrs)	89.12	78.01	-	5.1
Neutral (kept for 6hr)	86.22	89.90	-	-
H_2O_2 6% (kept for 6 hrs)	83.13	85.20	-	3.8
Heat dry (60^0C,6 hrs)	80.44	86.62	-	-
Photo stability	84.34	88.11	-	-

***Method Validation*:** validation parameters checked as per ICH Q2A (R1) guideline ICH Q2A (R1) (2005)

Specificity

The specificity of the method was ascertained by peak purity profiling studies. The peak purity values were found to be more than 980, indicating the non-interference of any other peak of degradation product, impurity or matrix.

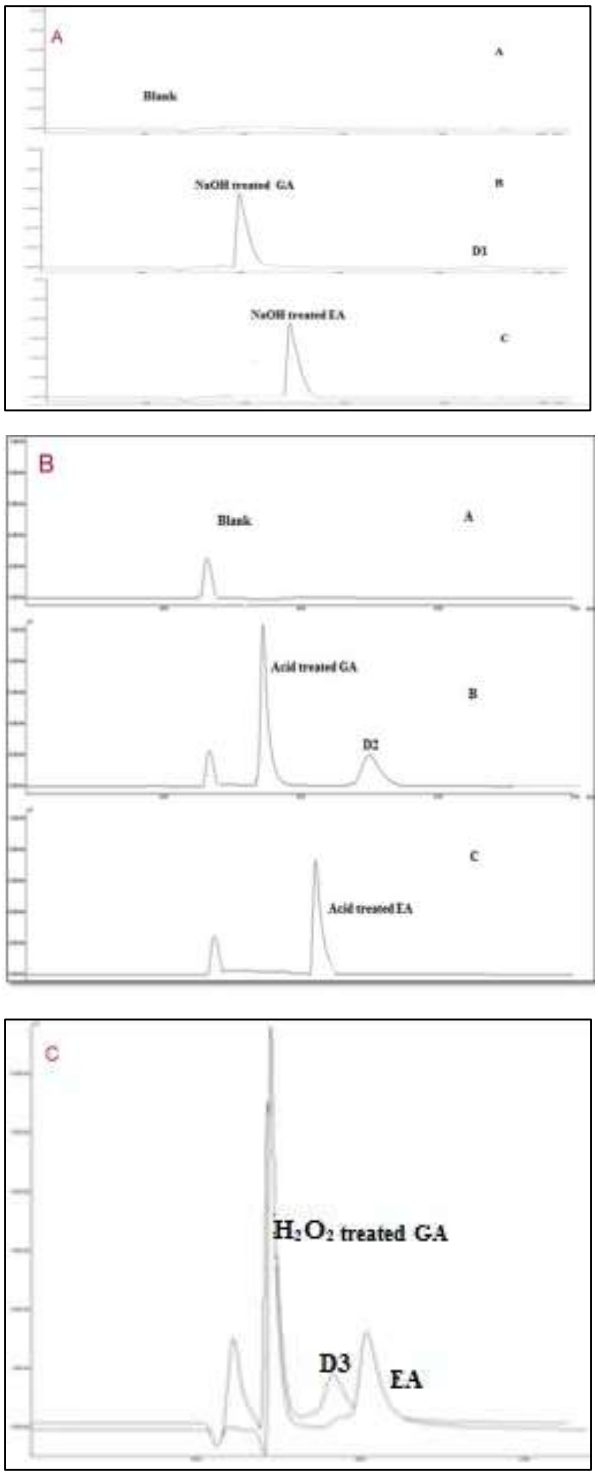

Fig.3: Chromatogram GA and EA, A) NAOH Treated B) Acid Treated C) Overlay of standard mix of GA & EA & H_2O_2 Treated GA

Linearity

From the standard stock solution (1000µg/ml) of EA and GA mixed standard solutions were prepared containing 8-24 µg/ml of each. These solutions were further used for

injection. Five replicates per concentration were injected (Table 3; Fig. 4)).

Calibration curve of EA and GA shown in Fig. 5.

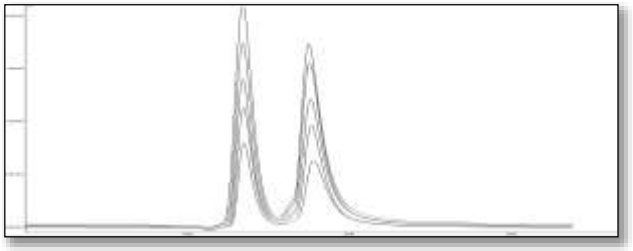

Fig. 4: Linarity of EA and GA

Table 3: Linarity of EA and GA

S.N.	Conc. (µg/ml.)	Peak Area	
		EA	GA
1	8	704158	407303
2	12	919925	695330
3	16	1152871	992526
4	20	1353671	1353671
5	24	1665007	1668774

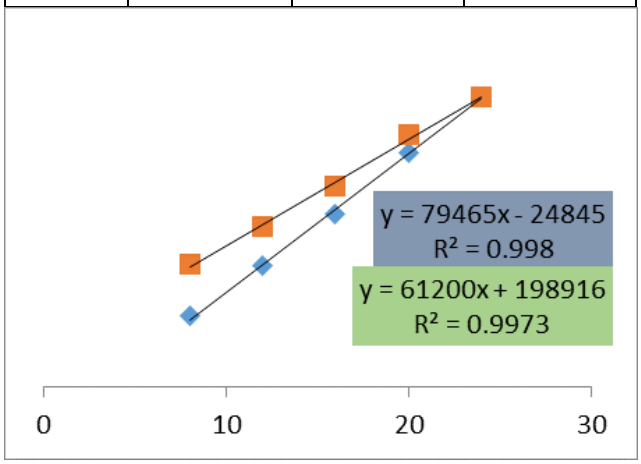

Fig. 5: Calibration curve of EA and GA

Precision

Inter-day precision

Precision of the system was evaluated by analyzing three independent standard preparations on three different days and % RSD value obtained was calculated to determine system precision. Result are tabulated in Table.4

Table.4: Inter-day

Conc. (µg/ml)	Mean Area		%RSD	
	EA	GA	EA	GA
8	701226	405996	0.35	0.88
12	923307	693495	1.32	1.26
16	1153896	993225	1.03	0.51

Intra-day precision

Precision of the system was evaluated by analyzing six independent standard preparations in a day and % RSD value obtained was calculated to determine system precision. Result are tabulated in Table.5

Table. 5: Intra-day precision

Replicate	Intraday (Mean Area)	
	EA	GA
1	701662	411949
2	712345	401876
3	703435	407546
4	701675	408567
5	701676	406578
6	704158	407303
Mean Area	704159	407303
% RSD	0.58	0.80

Accuracy

Accuracy studies were performed by adding 80%, 100%, and 120% solutions with respect to target assay concentration (10µg/ml)

Table .6: Recovery studies of EA and GA

Level %	Sample	Standard (µg/ml)	Mean Area		% Recovery	
			EA	GA	EA	GA
80	10	8	1310728	1344399	100.43	95.72
100	10	10	1459421	1515331	102.23	96.94
120	10	12	1569121	1653950	101.25	96.02

Limit of detection (LOD) and limit of quantification (LOQ)

The **LOD** of EA and GA = 0.25µg/ml, 0.15µg/ml .

LOQ= EA0.75µg/m, 0.49µg/ml. resp

Robustness

Robustness of the method was determined by carrying out the analysis under conditions during which wavelength, flow rate, mobile phase composition were altered and the effects on the area was noted. The results obtained are shown in Table.7

Table.7: Robustness study

Marker	% RSD Found For Robustness Study (peak area)								
	Wavelength			Flow Rate			Mobile phase 1% OPA:ACN(70:30)		
	270	271	272	0.9	1	1.1	72:28	70:30	68:32
EA	1.33	1.03	1.39	1.34	0.50	1.65	1.52	0.51	1.58
GA	1.07	0.84	1.69	1.06	0.99	1.48	1.6	0.99	1.07

Assay

This developed & validated method was applied for assay of jambasav sample. 5ml of jambasav was accurately measured. Transfered to 25ml beaker. 5 ml methanol was added in the beaker. Stirred well & Centrifuged at 1000rpm for 10 mins in test tube. The resultant solution was filtered through whattman filter paper & was used for injection. The results are tabulated in Table 9.

Table 8: Formulation details

Product Name	Manufacture	Mfg. Date	Expiry Date
Jambasav	Aphali Pharmaceuticals Ltd	3/2014	2017

Table 9: %Assay for EA and GA from jambasav

Area		% assay	
EA	GA	EA	GA
378941	776174	0.1612	0.2880

Conclusion

The developed method was found to be simple, time saving, economic, accurate and precise. This method can be used for monitoring stability of Ellagic and Gallic acid.

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