ISSN: 2322 - 0902 (P)
ISSN: 2322 - 0910 (O)

International Journal of Ayurveda and Pharma Research

Research Article

HPTLC ESTIMATION OF GALLIC ACID AND ELLAGIC ACID IN *AMRTOTTARA KVATHA* PREPARED IN TWO RATIOS

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ABSTRACT

Amrtottara kvatha is a decoction which is used primarily in the management of hyperpyrexia (*jwara*). Fresh stem of *Tinospora cordifolia* (Willd.) Miers (*Guduchi)*, dried fruit rind of *Terminalia chebula* Retz *(Haritaki)*, and dried rhizome of *Zingiber offficinale* Roscoe (*Shunti*) in the ratio 3:2:1 are its ingredients. From the point of view of drug design, it has the peculiarity that, its ingredients are specified to be added in a particular ratio, rather than in equal amounts as is the case in most of the compound formulations in Ayurveda. But, the rationale behind the drug design are not detailed enough to give much information regarding the effect of alteration of the drug ratio etc. Thus, in the present study an exploration was made with respect to its phytochemistry, by comparing with the decoction prepared in the general ratio of 1:1:1. The compounds Gallic acid and Ellagic acid were used for quantitative evaluation and comparison using HPTLC method. Estimation of Gallic acid in the samples showed that the amount of Gallic acid in *Amrtottara kvatha* prepared in classical ratio of 3:2:1 is significantly higher than that prepared in the altered ratio of 1:1:1 with a p value <0.05, though the amount by weight of *Haritaki* (source of Gallic acid) was unaltered in both, thus indicating the possibility of some complex phytochemical interactions among the constituents. With respect to Ellagic acid, there was no statistically significant difference in its quantity in the two decoctions compared. The method developed for HPTLC analysis in this study can be used as a technique for standardization of *Amrtottara kvatha*.

KEYWORDS: *Amrtottara kvatha*, Ratio, Gallic acid, Ellagic acid, HPTLC.

INTRODUCTION

Amrtottara Kvatha is an Ayurvedic formulation derived by the traditional Ayurvedic practitioners of Kerala. This decoction is one of the widely used preparations which is primarily indicated for hyperpyrexia (*Jwara*). The drugs used in its preparation are, fresh stem of *Tinospora cordifolia* (Willd.)Miers (*Guduchi)*, dried fruit rind of *Terminalia chebula* Retz. (*Haritaki*) and dried rhizome of *Zingiber offficinale* Roscoe. (*Shunti)* in the ratio 3:2:1.[1,2] From the point of view of drug design, this poly herbal formulation has the peculiarity that, its ingredients are specified to be added in a particular ratio, rather than in equal amounts as is the case in most of the compound formulations in Ayurveda.

The history of Ayurveda suggests that, drugs mentioned in the ancient textbooks of Ayurveda, including single herbs and compound formulations may be a result of a long period of clinical observations made by the scholars through ages. But, the rationale behind the design of these compound formulations are not detailed enough to give much information regarding the effect of alteration of the drug ratio etc. Thus it is a matter of interest to explore if the particular ratio of *Amrtottara kvatha* has any rationale, as it is different from the common practice of taking drugs in equal quantities. Such exploration was made with respect to its phytochemistry in the present study.

A total of ten compounds have been reported so far from *Amrtottara kvatha*. They are Gallic acid, Chebulic acid, Quinic acid, Tartaric acid, Protocatechuic acid-4-glucoside, Caffeoyl glucose, Quercetin, Quercetin-3- glucuronide, Quercetin rhamnoside and Ellagic acid.[3,4] In the present study, the compounds Gallic acid and Ellagic acid, the major compounds isolated from *Terminalia chebula* were used for analysis of the decoction. The pharmacological activities reported from Gallic acid include anti-inflammatory, immunomodulatory and anticarcinogenic.[5] Ellagic acid has activities like anti-inflammatory, anti-carcinogenic, anti-microbial, and anti-arthritic.[6]

In this study, Gallic acid and Ellagic acid were used as standards, and their quantities were compared in *Amrtottara kvatha* prepared in the original ratio of 3:2:1, and that prepared in altered ratio of 1:1:1 using HPTLC (High Performance Thin Layer Chromatography) method.

MATERIALS AND METHODS

Materials

Gallic acid, Ellagic acid

The compounds Gallic acid and Ellagic acid were isolated from acetone extract of *Haritaki* (*Terminalia chebula*) using repeated gravitational column chromatography, and characterized using nuclear magnetic resonance (NMR) and high resolution mass spectrometry (HRMS) analysis. These were used as standards for analysis.

Amrtottara kvatha

The decoctions *Amrtottara kvatha* (AMK 321) and *Amrtottara kvatha* in the altered drug ratio of 1:1:1 (AMK 111) were prepared in laboratory according to standard procedure.[7] The raw drugs for its preparation were procured from botanical garden, Govt. Ayurveda College Trivandrum (*Tinospora cordifolia*), Govt. Agricultural College, Vellayani (*Zingiber officinale*) and natural habitat from Kottoor, Trivandrum (*Terminalia chebula*). These were authenticated by botanist at Pharmacognosy unit, Govt. Ayurveda College Trivandrum.

Reagents

HPLC grade solvents from Merk, Germany were used.

Instruments and equipment used

High Performance Thin Layer Chromatograph (CAMAG, Switzerland) system, Linomat V sample applicator, photo documentation chamber (CAMAG Reprostar 3) with dual wavelength UV lamp (254 nm and 366 nm), CAMAG twin-trough chambers, CAMAG TLC scanner III, controlled by WINCATS software 1.3.4 version, analytical balance, and air blower.

Methods

Preparation of standard solution

Stock solution of Gallic acid and Ellagic acid were prepared by dissolving 11 mg of each of the compounds in 100 ml methanol (0.11mg/ml).

Preparation of AMK 321

3gm of cut and crushed fresh stem of *Tinospora cordifolia* (*Guduchi*), 2gm of coarsely powdered dried fruit rind of *Terminalia chebula* (*Haritaki*) and 1gm of coarsely powdered dried rhizome of *Zingiber officinale* (*Shunti*) were mixed together and boiled in 96ml water, and reduced to 12ml and strained through a clean 4 layered cotton cloth. (Table no. 1)

Preparation of AMK 111

2gm of cut and crushed fresh stem of *Tinospora cordifolia* (*Guduchi*), 2gm of coarsely powdered dried fruit rind of *Terminalia chebula* (*Haritaki*) and 2gm of coarsely powdered dried rhizome of *Zingiber officinale* (*Shunti*) were mixed together and boiled in 96ml water, and reduced to 12ml and strained through a clean 4 layered cotton cloth. (Table no. 1)

Table 1: Quantity of ingredients of AMK 321 and AMK 111

Sample decoction	Weight of *Guduchi*	Weight of *Haritaki*	Weight of *Nagara*
AMK 321 (classical preparation)	3 g	2g	1g
AMK 111(altered)	2g	2g	2g

Figure n1: Preparation of *Kvatha* (a) process of preparation (b) prepared *Kvatha* (AMK 321)

Extraction of Gallic acid and Ellagic acid from decoctions

10ml each of these decoctions were taken in glass beakers and kept on water bath maintained at 80^0C for about 2 hours until a dry residue was obtained. The residue was dissolved in 10ml methanol each by continuous stirring with a glass rod for 20 minutes. The undissolved portion was filtered off using Whatman filter paper, and the clear solutions were made up to 10ml in a 10ml volumetric flask. These were used for quantification.

Chromatographic conditions

Chromatographic separation was achieved on HPTLC plates (20×20cm) pre-coated with silica gel 60 F_{254} with aluminum sheet support. The plates were prewashed with methanol and activated at 110°C for 8 minutes. Standard solutions and the sample solutions were applied to the plates as 6.0mm wide bands, 10.0mm from the bottom edge of the chromatographic plate by using of a Camag (Switzerland) Linomat 5 sample applicator. The plate was developed with mobile phase to a height of 70mm in a Camag glass twin-trough chamber. After development, the plates were dried, viewed at 254 nm and 366 nm, and photographed. This was followed by densitometric scanning with a Camag TLC Scanner 3 using Wincats software. Spectral scanning was done at the wavelength of maximum absorbance.

Mobile phase development

The standard stock solution of Gallic acid and Ellagic acid were spotted on to separate TLC plates and developed in different solvent systems. The mobile phase developed for Gallic acid was, Toluene, Ethyl acetate and Formic acid in the ratio 5:7:1. That for Ellagic acid was, Toluene, Methanol, Ethyl acetate and Formic acid in the ratio 4:1:4:1.

Quantification

Quantification of Gallic acid and Ellagic acid in the samples (AMK 321 extract and AMK 111 extract) were done separately.

For the purpose of quantification of Gallic acid, six different concentrations of standard Gallic acid and the two samples, viz., AMK 321 extract and AMK 111 extract were applied on TLC plate simultaneously. The volumes of standard and sample which give a desirable calibration curve was selected for estimation. The volumes of standard used were, 3µl, 5µl, 7µl, 9µl, 11µl, 13µl (i.e., 0.33µg, 0.55µg, 0.77µg, 0.99µg, 1.21µg, 1.43µg) of standard stock solution. Samples were applied in the volume of 4µl. For obtaining calibration curve, densitometric scanning of the plate was performed in absorbance mode at the wavelength of maximum absorbance. The wavelength of maximum absorbance was found to be 278nm for Gallic acid. Quantitative data was obtained from the software by fixing the percentage deviation to get an appropriate regression line having a desirable standard deviation (<5) and regression coefficient (<1), while including sample spots in the regression line. Percentage weights by weight (%w/w) of Gallic acid in samples were calculated with respect to the % weight of drug in *Kvatha*.

For the quantification of Ellagic acid in samples, same method was used, except for the change in the sample volume. Here 2µl of samples were applied. The wavelength of maximum absorbance was found to be 278nm for Ellagic acid.

Validation of the HPTLC method

Specificity

The specificity of the method was ascertained by applying standard and blank simultaneously on the TLC plate. The spot of Ellagic acid was confirmed by comparing Rf value and spectra of the spot with that of standard. The peak purity of standard was assessed by comparing the spectra at three different levels, i.e. peak start, peak apex and peak end positions of the spot.

Precision

Instrumental precision was measured by replicate (n=6) application of same standard solution.

Linearity

Linearity was evaluated by visual inspection of a plot of signals as a function of analyte concentration.

Accuracy

It was inferred after precision, linearity and specificity were established.

Detection limit

Limit of detection= $3.3\sigma/ S$; where σ = the standard deviation, S = the slope of calibration curve

Quantification limit

Limit of quantification=$10\sigma/S$

RESULTS

Gallic acid estimation

TLC at 254nm (Photo documentation)

Under 254 nm, both the samples showed 2 spots. No visible spots were seen at 366nm. (Figure no. 2 A)

Densitometric scanning

Densitometric scanning at 254nm gave peaks of Gallic acid (GA) at an Rf value 0.45±0.005. The Rf values of the peaks obtained were, GA 1- 0.45, GA 2- 0.45, GA 3- 0.45, GA 4- 0.46, GA 5- 0.45, GA 6- 0.45, AMK 321- 0.46, 0.65, 0.91, AMK 111- 0.45, 0.66, 0.73, 0.91.

Spectral comparison of standard and samples (at 278nm)

Based on the initial spectral scanning, the spectral comparison was done at 279nm, which was the wavelength of maximum absorbance. The spectra of standard and sample were super imposable.

Quantification

A linear calibration curve was obtained both via peak area and peak height. The standard deviations and regression coefficients were desirable. Three replicate analysis were done for statistical analysis. The regression equation (via peak area), Y= 3177+5.617X showed a standard deviation of 1.18%

and a regression coefficient 0.9989. (Figure no. 3 D) The amount of Gallic acid in samples was calculated based on the quantification of Gallic acid in the Wincats software, for the respective regression equations. It is expressed as %w/w of *Kvatha* and given in the following table.

Table 2: % w/w of Gallic acid in *Kvatha* samples

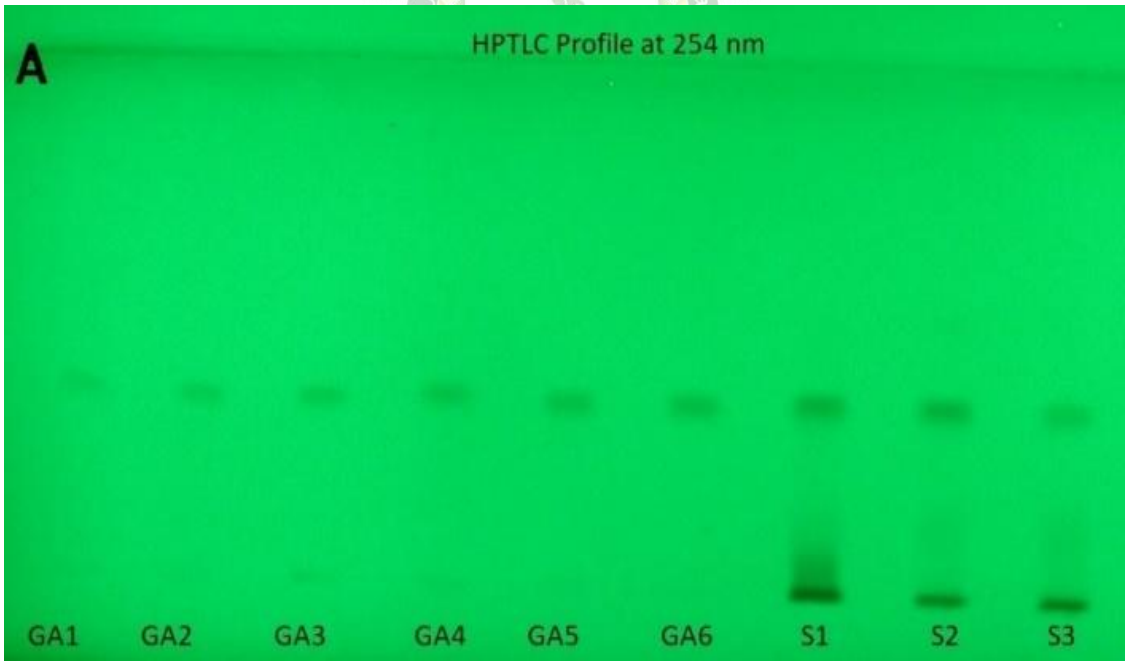
S.No.	Sample	%w/w of Gallic acid (Mean±SD)
2.	AMK 321	0.104 ±0.000
3.	AMK 111	0.053 ±0.001

Validation studies

The results of validation studies have been tabulated below:

Table 3: Results of validation studies for method for Gallic acid quantification

S.No.	Experiment	Observation	Result
1.	Specificity	Rf of spot both in the standard and the sample were approximately same, with an overlapping spectra	Specific
2.	Instrumental precision	Rf of the spots= 0.45±0.004	Precise
3.	Linearity	Linear calibration curve was obtained both via height and area	Linear
4.	Accuracy	Inferred to be accurate as it was validated for precision, linearity and specificity	Accurate
5.	Limit of detection	-	0.69ng
6.	Limit of quantification	-	2.1ng



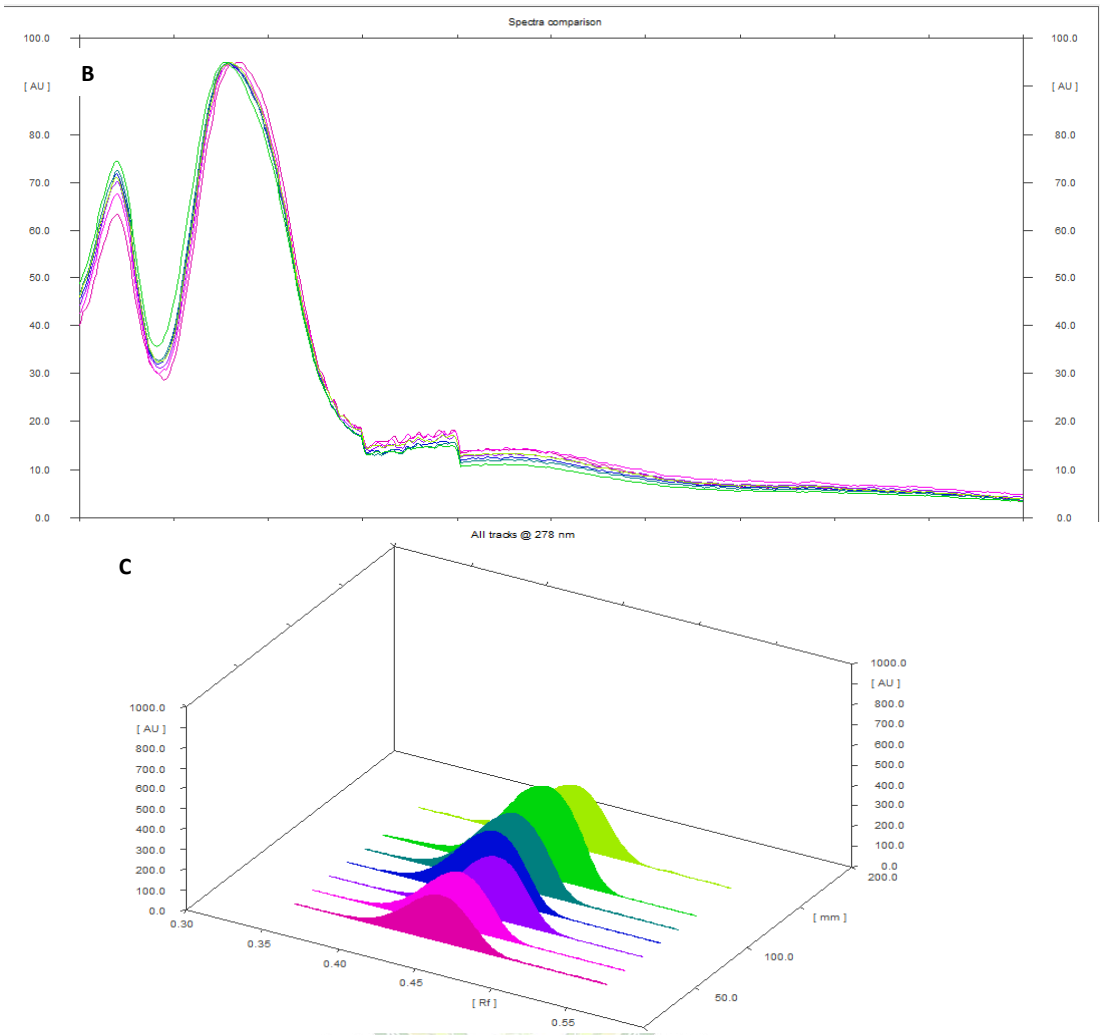


Figure 2A: HPTLC profile of standard and samples at 254nm; GA 1- GA 6: standard Gallic acid solution in different concentrations; S1-AMK 321extract, S3-AMK 111extract, **2B.** Spectral comparison of standard Gallic acid and Gallic acid quantified from samples; **2C.** 3D display of chromatogram of standard Gallic acid and Gallic acid quantified from samples.

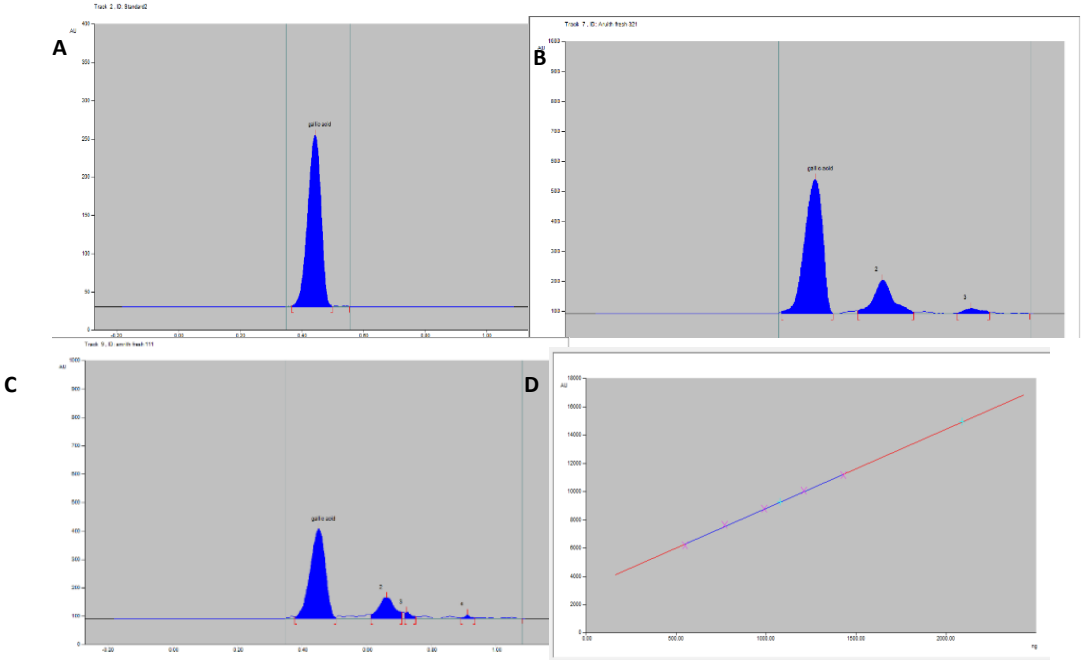


Figure 3A: Peak display of standard Gallic acid; **3B.** peak display of AMK 321extract; **3C.** peak display of AMK 111extract; **3D.** Calibration plot of Gallic acid via peak area

Statistical analysis

The collected data on quantity of Gallic acid from groups AMK 321 and AMK 111 were subjected to analysis using appropriate statistical tools. All the data were represented as mean ± SD. Since the data distribution is normal, independent sample t test was used for comparing AMK 321 and AMK 111 based on quantity of Gallic acid. A calculated P value < 0.05 is considered to be statistically significant. All the analysis was done with the help of software SPSS version 22.0 for windows.

Table 4: Data and test of significance (t test) for comparison of AMK 321 and AMK 111

Compound	Group	Mean (% w/w)	SD	t	P value
Gallic acid	AMK 321	0.1044	0.00006	127.143	0.000*
	AMK 111	0.0534	0.00069		

* Significant (p<0.05)

From table, independent sample t test showed that there exists significant difference in average quantity of Gallic acid between AMK 321 and AMK 111 (t =127.143, P < 0.05). AMK 321 reported significantly greater quantity of Gallic acid (0.1044± 0.00006 % w/w) as compared to AMK 111(0.0532 ± 0.00069 % w/w).

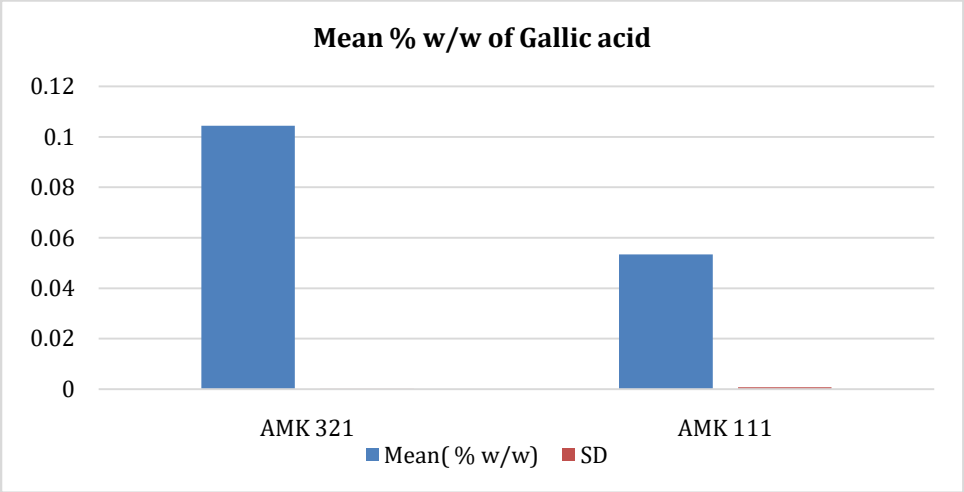


Figure 4: Bar diagram of mean value of % w/w of Gallic acid in AMK 321 and AMK 111 extracts

Ellagic acid estimation

TLC at 254nm (Photo documentation)

Under 254nm, 2 spots were observed for both the samples (Figure no. 5 A)

Densitometric scanning

Densitometric scanning at 254nm gave peaks of Ellagic acid (EA) at an Rf value 0.39 ± 0.0038. The Rf values of the peaks obtained were, EA 1- 0.39, EA 2-0.39, EA 3-0.39, EA 4-0.39, EA 5-0.39, EA 6-0.39, AMK 321extract-0.40, 0.66, AMK 111 extract- 0.40, 0.67.

Spectral comparison of standard and samples (at 279nm)

Based on the initial spectral scanning, the spectral comparison was done at 279nm, which was the wavelength of maximum absorbance. The spectra of standard and sample were super imposable.

Quantification

A linear calibration curve was obtained both via peak area and peak height. The standard deviations and regression coefficients were desirable. Three replicate analysis were done for statistical analysis. The regression equation (via peak area), Y= 1891.7+14.7X showed a standard deviation of 2.32% and a regression coefficient 0.9976 (Figure no. 6 D). The amount of Ellagic acid in samples were calculated based on the quantification of Ellagic acid in the Wincats software, for the respective regression equations. It is expressed as %w/w of *Kvatha* and given in the following table.

Table 5: % w/w of Ellagic acid in *Kvatha* samples

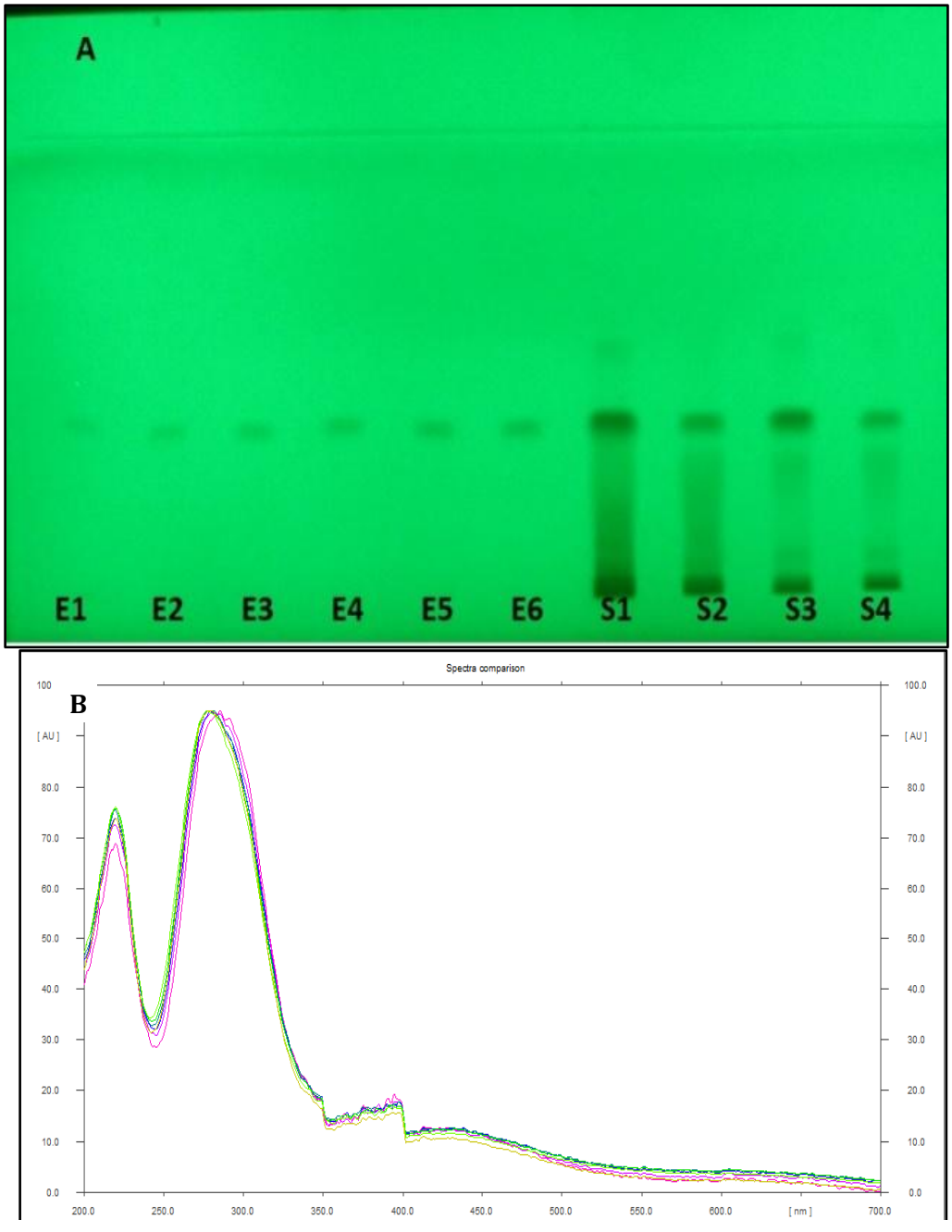
S.No.	Samples	%w/w of Ellagic acid (Mean±sd)
2.	AMK 321 extract	0.050±0.016
3.	AMK 111 extract	0.039±0.011

Validation studies

The results of validation studies have been tabulated below

Table 6: Results of validation studies for method for Ellagic acid quantification

S.No.	Experiment	Observation	Result
1.	Specificity	Rf of spot both in the standard and the sample were approximately same , with an overlapping spectra	Specific
2.	Instrumental precision	Rf of the spots= 0.39±0.000	Precise
3.	Linearity	Linear calibration curve was obtained both via height and area	Linear
4.	Accuracy	Inferred to be accurate as it was validated for precision, linearity and specificity	Accurate
5.	Limit of detection	-	0.52 ng
6.	Limit of quantification	-	1.577 ng



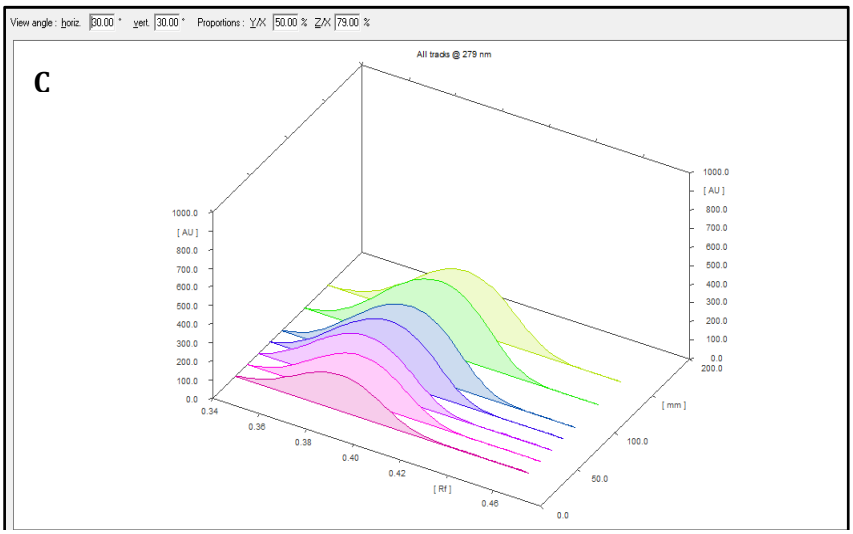


Figure no. 5A: HPTLC profile of standard and samples at 254nm, E 1-E6: standard Ellagic acid solution in different concentrations, S2-AMK 321 extract, S4-AMK 111 extract; **5B.** Spectral comparison of standard Ellagic acid and Ellagic acid quantified from samples; **5C.** 3D display of chromatogram of standard Ellagic acid and Ellagic acid quantified from samples

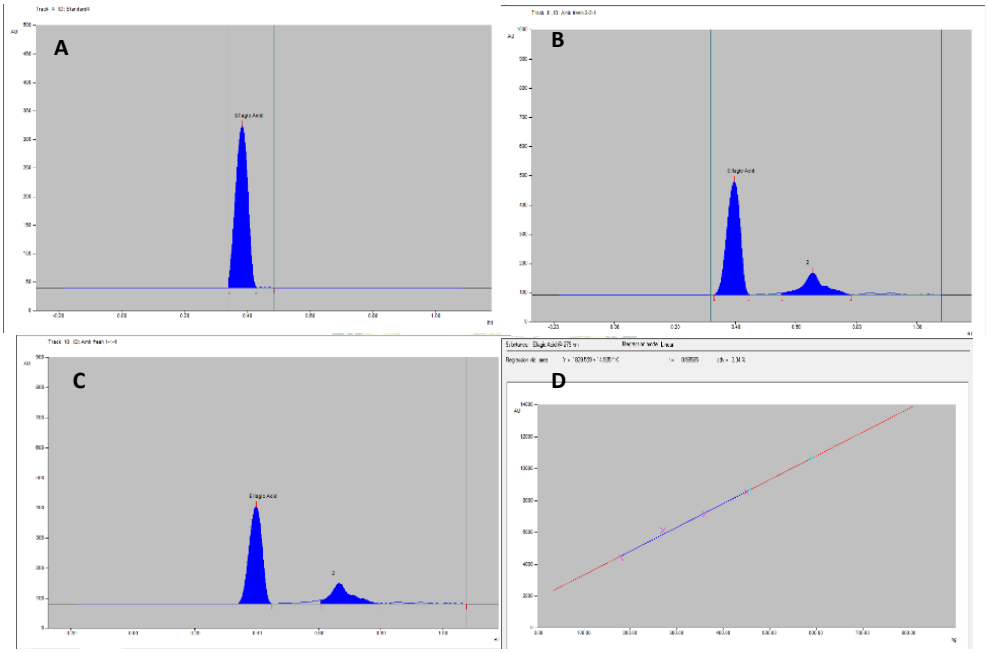


Figure no. 6A: Peak display of standard Ellagic acid; **6B:** peak display of AMK 321 extract; **6C.** peak display of AMK 111 extract; **6D.** Calibration plot of Ellagic acid via peak area

Statistical analysis

All the data on quantity of Ellagic acid were represented as mean ± SD. Independent sample t test was used for comparing AMK 321 and AMK 111 based on quantity of Ellagic acid. A calculated P value < 0.05 is considered to be statistically significant. All the analysis was done with the help of software SPSS version 22.0 for windows.

Table 7: Data and test of significance (t test) for comparison of Ellagic acid in AMK 321 and AMK 111 extracts

Compound	Group	Mean (%w/w)	SD	t	P value
Ellagic acid	AMK 321	0.0502	0.1619	0.942	0.399^{NS}
	AMK 111	0.0394	0.01137		

NS: Not significant (p> 0.05)

From table, independent sample t test showed that there is no significant difference in average quantity of Ellagic acid between AMK 321 and AMK 111(t =0.942, P >0.05). AMK 321 reported approximately the same quantity of Ellagic acid when compared to AMK 111.

DISCUSSION

Estimation of Gallic acid in the samples showed that the amount of Gallic acid in AMK 321 is significantly higher than that in AMK 111 with a p value <0.05. Here the percentage of Gallic acid in AMK 321 was approximately double than that in AMK 111. This is an important finding, as in both these ratios, the amount by weight of *Haritaki*, which is the source of Gallic acid was unaltered. This is due to some complex interaction between the phytochemicals. Estimation of Ellagic acid in samples showed that, there is no statistically significant difference in the quantity of Ellagic acid in AMK 321 and AMK 111. This is as expected, as the amount of *Haritaki* in *Amrtottara kvatha* prepared in these two ratios are the same.

CONCLUSION

Two major compounds isolated from *Terminalia chebula (Haritaki)* were used for analysis. HPTLC method was developed for the estimation of these compounds, Gallic acid and Ellagic acid in *Amrtottara kvatha*. From this study, it was revealed that, the quantity of Gallic acid is approximately double in classical *Amrtottara kvatha* prepared in 3:2:1 when compared to that prepared in 1:1:1, though the amount by weight of *Haritaki* (source of Gallic acid) was unaltered in both. The method developed for HPTLC analysis in this study can be used as a technique for standardization of *Amrtottara kvatha*.

AKNOWLEDGEMENT

Authors are thankful to Dr.K.V.Radhakrishnan, senior principal scientist, Organic chemistry section, CSIR-NIIST, Trivandrum, Mr.Mahadevan, Former Research Assistant, and Dr. Binitha Raj, research officer, Drug standardization unit, Government Ayurveda College, Trivandrum.

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Cite this article as:
R.S. Karthika, A. Shahul Hameed, M.T. Meenu. Hptlc Estimation of Gallic Acid and Ellagic Acid in Amrtottara Kvatha Prepared in Two Ratios. International Journal of Ayurveda and Pharma Research. 2019;7(8):1-9.
Source of support: Nil, Conflict of interest: None Declared

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