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Effect of Storage Condition on Polyphenol Content of *Emblica Officinalis, Terminalia Belerica* and *Terminalia Chebula*

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ABSTRACT

Accelerated stability studies of *Emblica officinalis, Terminalia belerica and Terminalia chebula* have been carried out as per ICH guidelines and its effect on total polyphenol content as determined by Folin-Ciocalteu method and Gallic acid content as determined by HPLC (High Performance Liquid Chromatography) was studied. The samples were kept in stability chamber at 40°C and 75% relative humidity for 3 months for accelerated stability studies. Samples were taken out at periodic intervals and extracted to determine total polyphenol content by spectrophotometric method and gallic acid content by HPLC. The HPLC method was also validated to demonstrate its selectivity, linearity, precision, and accuracy. The results indicate an increase in total polyphenolic content as well gallic acid content under accelerated stability conditions which is indicative of hydrolysis of gallotannic acids present in crude drugs to liberate free gallic acid, thereby increasing the total polyphenolic content.

Keywords: HPLC; polyphenols; gallic acid; *Emblica officinalis; Terminalia belerica; Terminalia chebula*

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INTRODUCTION

Since ancient time medicinal plants have been considered as main ingredients in Ayurvedic formulations for the treatment of various diseases and they have never lost their importance even with the emergence of modern science. An important part of quality control of herbal products is the evaluation of the chemical stability of a finished product during the storage period. Measuring chemical stability is very challenging task due to the complexity of a plant extract, which may contain thousands of different compounds. The purpose of stability testing is to provide evidence on how the quality of herbal products varies with the time under the influence of environmental factors, especially temperature, humidity, and oxygen and enables recommended storage conditions, retest periods and shelf lives to be established.[1]

E. officinalis, T. belerica and *T. chebula* are highly regarded as a universal panacea in the Ayurvedic medicine because of wide spectrum of medicinal activities. *E. officinalis* (*Amla*) primarily contains tannins (gallic acid, ellagic acid, chebulinic acid and chebulagic acid) isostrictinin, quercetin, amino acids and carbohydrates. The fruit of the plant is reported to possess significant antioxidant, immunomodulatory, antipyretic, analgesic, cytoprotective, antitussive and gastroprotective activity. Amla berries have the highest amount of naturally occurring vitamin C of any ripe fruit in the world used as a traditional food.[2,3,4,5]

T. chebula (*myrobalan*) fruits are also a rich source of hydrolysable tannins. The chief constituents of tannin are chebulic acid, chebulagic acid, corilagin and gallic acid. Myrobalan is reported to possess strong antioxidant activity, effect of which is attributed to the presence of phenolic constituents.[6,7,8] Other important pharmacological activities of the drug reported are adaptogenic, hypocholesterolemicacivity, anti-ulcerogenic, purgative, immunomodulatory and antimicrobial activity.[9,10]

The fruits of *T. belerica* (*Bibhitaki* or *Behada*) contain mainly polyphenols (gallic acid, ellagic acid, phyllembin, ethyl gallate, and chebulagic acid). Other constituents are triterpenoids including belleric acid, β-sitosterol, and the saponin glycosides bellericoside and bellericanin and lignans. The fruit possesses antibacterial, antimutagenic, antimalarial and antifungal properties and is employed in dropsy, piles and diarrhea.[11,12,13]

E. officinalis, T. belerica and *T. chebula* are important plant species well known for their antioxidant effects, mainly because of polyphenolic constituents and are widely exploited on commercial level for their numerous medicinal uses.[14] The present study is focused on changes in

total polyphenolic content and gallic acid content of *E. officinalis*, *T. belerica* and *T. chebula*, when subjected to accelerated stability studies.

MATERIALS AND METHOD

Plant material:

Authenticated plant material was purchased from Yucca Enterprises, Mumbai (MH).

Chemicals and reagents:

All the chemicals used for total polyphenolics estimation were of analytical grade and HPLC grade solvents were used for HPLC analysis were from SD Fine (Mumbai, India). HPLC grade gallic acid (≥99%) was purchased from Sigma-Aldrich (Mumbai, India).

Stability studies of crude plant drugs:

Crude plant materials of *E. officinalis*, *T. belerica* and *T. chebula* were kept in thermostability chamber (Model Innovative DTC-968, Dolphin, India) at 40°C and 75% relative humidity for accelerated studies out as per the ICH guidelines. Samples were taken out at periodic intervals (0, 1 and 3 Month) for analysis.

Extraction:

Dried powdered plant material of *E. officinalis*, *T. belerica* and *T. chebula* were taken out from stability chamber at periodic intervals. Accurately about one gram of each sample was extracted with water used in small portions. The sample solution was filtered to obtain a clear solution. The stock solution after suitable dilutions was used for further analysis.

Chromatographic conditions:

High performance liquid chromatography (HPLC) was performed using Phenomex C18 column (250*4.5 mm, 0.5 µ) on LC-20 AD Prominence Liquid chromatograph (Shimadzu, Japan) attached with Spd-20A/20AV Prominence SPD-20A prominence UV/Vis detector.

Determination of total polyphenolic content:

The Folin-Ciocalteu reagent is sensitive to reducing compounds including polyphenols, thereby producing a blue color upon reaction. This blue color is measured spectrophotometrically which is an indicative of total phenolic content. A double beam spectrophotometer 2203 from Systronics, Japan was used.

The total polyphenolic content of the aqueous fruit extracts of all three crude drugs were determined by UV spectrophotometry using Folin-Ciocalteu reagent.[15,16] Gallic acid was used as a standard and the total phenolics were expressed as gallic acid equivalents. Concentrations of 2, 4, 6, 8 and10 µg/ml of gallic acid were prepared in distilled water. The standard solutions were mixed with 1 ml diluted Folin-Ciocalteu reagent (1:1) and 1 ml of saturated sodium carbonate solution in

a 10 ml volumetric flask. The solutions were allowed to stand for 30 minutes at room temperature before the absorbance was read at 750 nm spectrometrically. Different concentration of aqueous extracts of *E. officinalis, T. belerica* and *T. chebula* (taken out at periodic intervals of 0, 1 and 3 Months) were separately treated in a similar way with Folin-Ciocalteu reagent. The change in the total polyphenolic content at different time interval was calculated and expressed in terms of gallic acid equivalent (GAE). All determinations were performed in triplicate.

Determination of Gallic acid content by HPLC

Quantitative determination of gallic acid was performed for each crude drug at different periodic intervals by HPLC as per the standard procedure cited in the literature with some modifications.[17,18,19] The chromatographic conditions were as follows: flow rate, 1.0 ml/min, volume injected 20 μL; detector was set at 280 nm (λ_{max} of gallic acid). The mobile phase composition was water: acetonitrile (80:20) containing 0.1 % *o*-phosphoric acid.

The retention time of the peak in the samples were compared with the standard used. The amount of gallic acid in crude drug extracts were determined from linear regression equation of calibration graph plotted between concentration (range of 2-20 μg/ml) and area of standard gallic acid.

METHOD VALIDATION

The optimized chromatographic method was validated according to the procedure described in ICH guidelines Q2 (R1) for the validation of analytical methods.

Linearity:

The linearity of calibration curve in gallic acid standard solution over the concentration range of 2-20 μg/ml through proposed HPLC method was carried out. Results were subjected to regression analysis.

Limit of detection and limit of quantification:

Limit of detection and limit of quantification were determined by using the formula based on the standard deviation of the response and the slope. LOD and LOQ were calculated by using equations, LOD= 3.3* σ/slope and LOQ = 10* σ/slope, Where σ= standard deviation

Precision:

The precision of the method was determined by repeatability, inter-day and intra-day reproducibility experiments of the proposed method. All the samples were analyzed in triplicate and mean was calculated.

Accuracy:

The accuracy of the proposed method was determined by recovery study, which was carried out by adding standard gallic acid in all three crude drugs. The pre-analyzed samples were spiked with

three different amounts of standard gallic acid prior to extraction. The spiked samples were extracted in triplicate and analyzed under the previously established optimal conditions.

RESULTS AND DISCUSSION

Total Phenolic Content:

Phenolic compounds have been proved to be responsible for the bioactivity of *E. officinalis, T. belerica* and *T. chebula*. The effect of storage conditions on amount of total phenolics in aqueous extracts of *E. officinalis, T. belerica* and *T. chebula* was measured in this study.

Total phenolic content in *E. officinalis* was found to be 27.05, 28.26 and 29.75 % on 0 day, 1 month and 3 month respectively when kept under accelerated storage conditions as per the ICH guidelines. For *T. chebula* the total polyphenolic content was found to be 55.95, 57.42 and 58.25% on 0 day, 1 month and 3 month respectively. *T. belerica* has shown a total polyphenolic content of 32.23, 34.45, 36.82 % on 0 day, 1 month and 3 month respectively when kept under accelerated storage conditions.

The results suggests that there has not been any decrease in total polyphenolic content in either of the crude drug tested, rather there it has been found that total polyphenolic content has increased to some extent in all tested crude drug and an increase of 14.24 % is found in case of *T. belerica.* The change in polyphenolic content is may be an indicative of hydrolysis of some of gallotannic acid present in crude drugs to liberate free gallic acid, thereby increasing the total polyphenolic content.

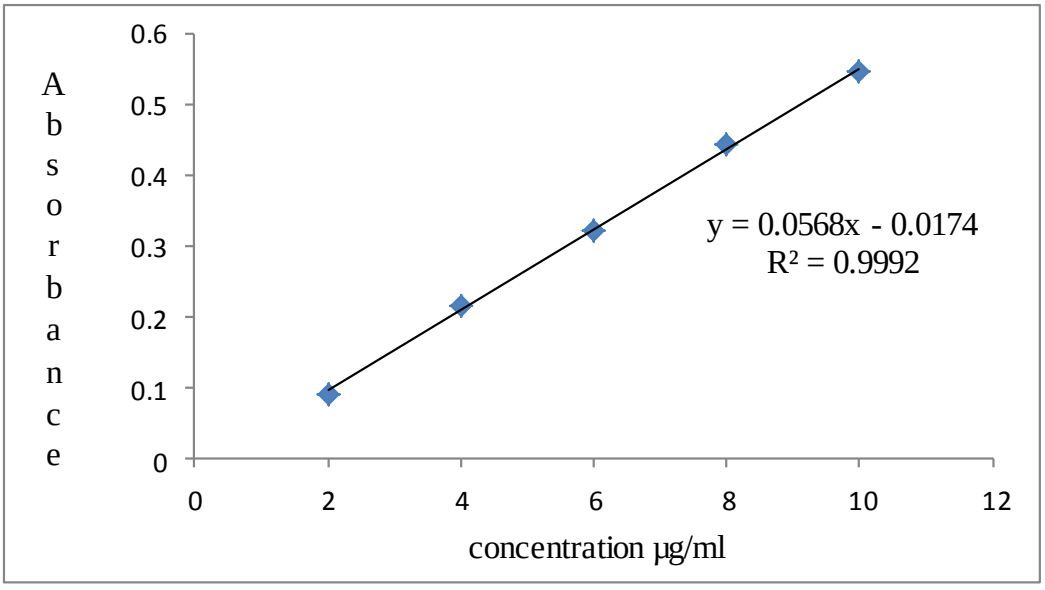

Figure 1: Calibration graph of gallic acid for total polyphenolics as determined by Folin-Ciocalteu method

Table 1: Total polyphenolic content in crude drugs as determined by Folin-Ciocalteu method under accelerated storage condition

S.No.	Crude drug	Time interval	Total polyphenolics (% GAE)	% Change in Gallic acid
1.	*E. officinalis*	0 day	27.05	--
		1 Month	28.26	4.47
		3 Month	29.75	9.98
2.	*T. chebula*	0 day	55.95	--
		1 Month	57.42	2.62
		3 Month	58.25	4.11
3.	*T. belerica*	0 day	32.23	--
		1 Month	34.45	6.82
		3 Month	36.82	14.24

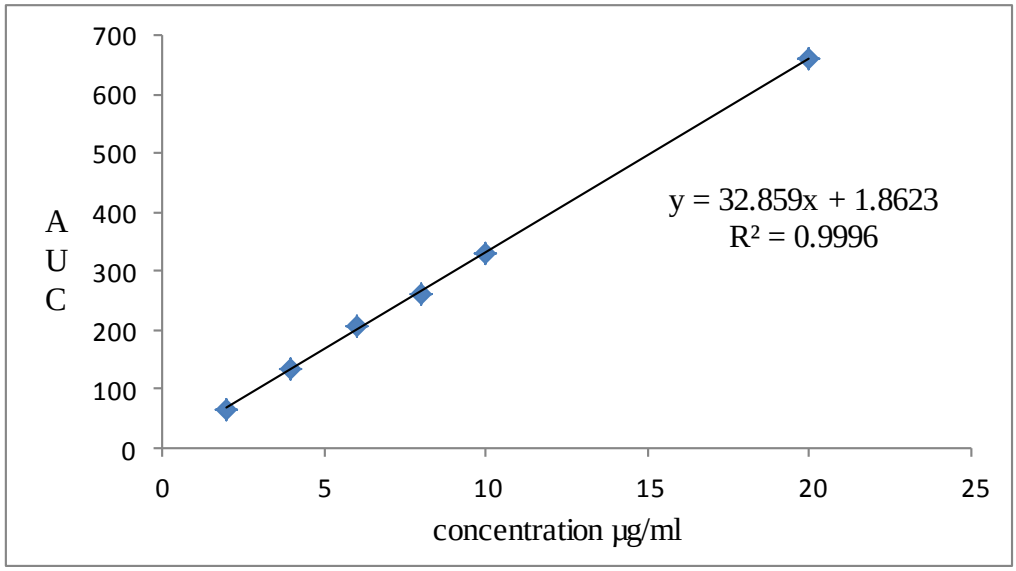

Figure 2: Calibration graph of gallic acid by High Performance Liquid Chromatography

Table 2: Regression parameters, linearity, limit of detection and limit of quantification of gallic acid

Drug	Conc. Range (µg/ml)	RT(min)	Regression equation	R^2	LOD (µg/ml)	LOQ (µg/ml)
Gallic acid	2-20	3.5	y=32.859x+1.8623	0.9996	0.45	1.364

Gallic Acid Content by HPLC

In the present study, gallic acid content in *E. officinalis, T. belerica* and *T. chebula* was determined on 0 day, 1 Month and 3 Month when kept under accelerated storage conditions as per the ICH guidelines by HPLC. The optimized isocratic HPLC conditions at 280 nm were used for the present study. The method was found to be linear in the range of 2-20 µg/ml with a correlation coefficient of 0.9996. The LOD and LOQ of gallic acid were found to be 0.45 and 1.36 µg/ml

respectively. The low % RSD values of for inter-day and intra-day variation reveal that the proposed method is precise (Table 3). The high recovery values (98.02-100.77) indicate a satisfactory accuracy (Table 4). Therefore the present HPLC method can be regarded as accurate and precise for the estimation of gallic acid in *E. officinalis, T. belerica* and *T. chebula.*

Table 3: Precision of inter-day and intra-day HPLC measurement for gallic acid in crude drugs

Drug	Intra-day[b] Content[a] (%Gallic acid)	% RSD	Inter-day[c] Content	% RSD
E. officinalis	3.982 ± 0.02	0.98	3.974 ± 0.02	0.93
T. chebula	5.963 ± 0.04	0.96	5.970 ±0.05	1.02
T. belerica	4.928 ± 0.03	0.95	4.932 ±0.05	0.95

[a]= Mean ± RSD

[b]= Samples were analyzed three times a day

[c]= Samples were analyzed once a day over three consecutive day

Table 4: Repeatability and recovery test for gallic acid in crude drugs

Drug	Gallic acid Content (µg/ml)	Amount added(µg/ml)	Recorded Amount	% Recovery
E. officinalis	3.982	2.0	5.941	99.31
		6.0	9.865	98.82
		10.0	13.931	99.63
T. chebula	5.963	2.0	7.966	99.96
		6.0	12.056	100.77
		10.0	15.647	98.02
T. belerica	4.928	2.0	6.797	98.10
		6.0	10.979	100.46
		10.0	14.875	99.64

Gallic acid content in *E. officinalis* was found to be 3.982, 4.123, 4.741 % on 0 day, 1 month and 3 month respectively when kept under accelerated storage conditions as per the ICH guidelines. For *T. chebula* the gallic content was found to be 5.963, 6.132 and 6.638 % on 0 day, 1 month and 3 month respectively. *T. belerica* has shown a gallic acid content of 4.928, 5.102, 5.221 % on 0 day, 1 month and 3 month respectively when kept under accelerated storage conditions. There has not been any significant change found in gallic acid content over the period of 1 Month when kept under accelerated storage conditions, while an increase in free gallic acid content in all the three drugs was observed over the period of three months. The result is in positive correlation with the total phenolic content as determined by spectrophotometry and is probably an indicative of hydrolysis of gallotannic acid to liberate free gallic acid. A study by Kim *et.al* has also reported an

increase in antioxidant and anti-microbial activity of tannic acid due to liberation of free gallic acid on hydrolysis of tannic acid.[20]

Table 5: Gallic acid content in crude drugs as determined by HPLC under accelerated storage condition

S.No.	Crude drug	Time interval	Gallic acid content (%)	% Change in Gallic acid
1.	*E. officinalis*	0 day	3.982	--
		1 Month	4.123	3.54
		3 Month	4.741	14.03
2.	*T. chebula*	0 day	5.963	--
		1 Month	6.132	2.83
		3 Month	6.638	11.32
3.	*T. belerica*	0 day	4.928	--
		1 Month	5.102	3.53
		3 Month	5.221	5.94

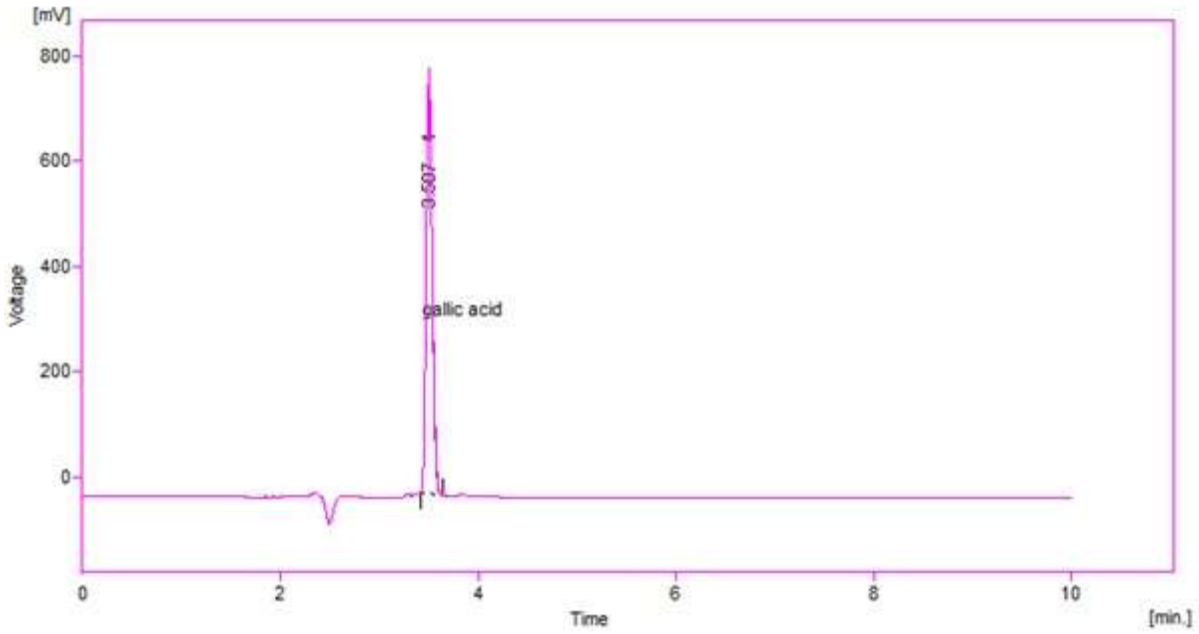

Figure 3: HPLC chromatogram of standard gallic acid

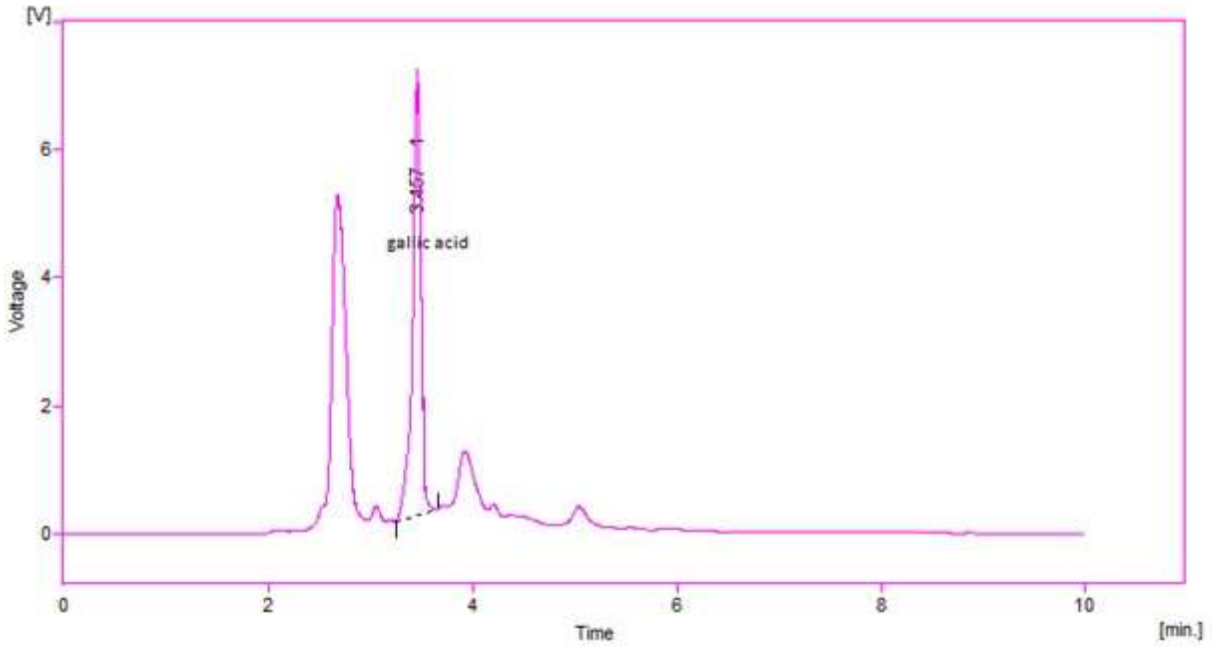

Figure 4: HPLC chromatogram of *E. officinalis*

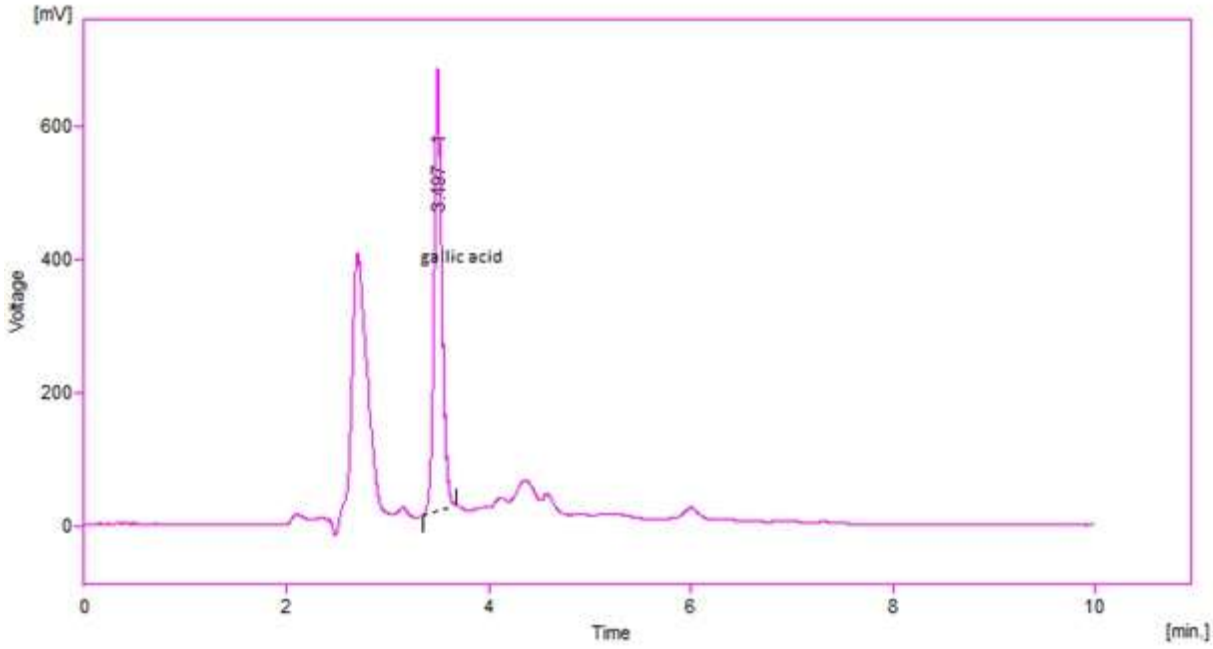

Figure 5: HPLC chromatogram of *T. chebula*

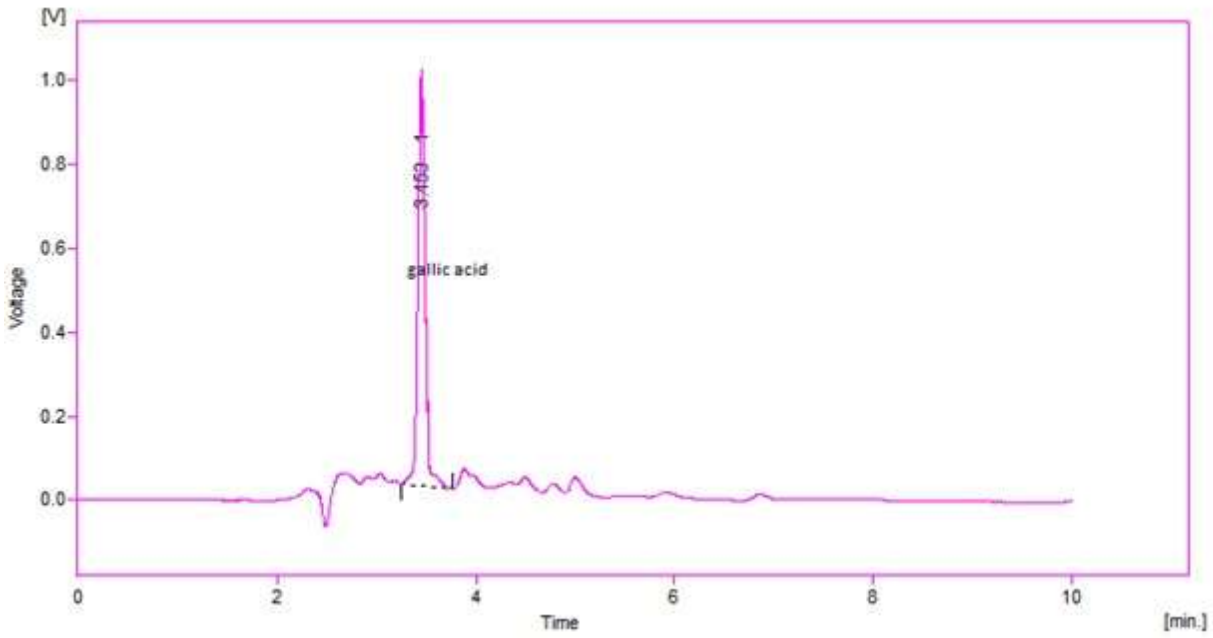

Figure 6: HPLC chromatogram of *T. belerica*

CONCLUSION

The total phenolic content and gallic acid content of aqueous extracts of *E. officinalis, T. chebula and T. belerica* was analyzed when stored under accelerated storage conditions as per the ICH guidelines. Total phenolic content when analyzed has shown an increase in all the tested crude drugs. Gallic acid content by HPLC was determined over the period of accelerated storage conditions and an increase in free gallic acid content was observed in all three crude drugs at 3 month interval. However a more detailed investigation on individual gallotannic acid and its degradation product needs to be carried out. Outcome of the study suggest a stable physical, chemical and biological nature of *E. officinalis, T. belerica* and *T. chebula* supporting the traditional claims of long term usage of these drugs for treatment of various ailments.

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