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HPLC Determination of the Gallic Acid and Chebulinic Acid Contents of *Phyllanthus emblica* Linn., *Terminalia bellirica* Roxb., *Terminalia chebula* Retz. and Triphala Products from Chae Son district, Lampang, Thailand

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Abstract The gallic acid and chebulinic acid contents in *Phyllanthus emblica* Linn., *Terminalia bellirica* Roxb., *Terminalia chebula* Retz. and Triphala products from Chae Son district, Lampang, Thailand at 2017 and 2018 seasons were studied. The HPLC separation was validated for analysis the hexane, ethyl acetate and methanol herb extracts. The gallic acid contents in the methanol extracts of *P. emblica* and *T. chebula* collected in 2018 were amounted to be double those of the same plants collected in 2017. The chebulinic content in the methanol extract of *P. emblica* collected in 2018 was almost double that of the same plant collected in 2017. The gallic acid contents of two Triphala products (with sugar and sugar-free) were not found to be different while the chebulinic contents of the two Triphala products for 2018 were 50% higher than those of 2017. The gallic acid and chebulinic acid contents variation form year to year should be taken into consideration for the production of Triphala products.

Keywords: *Triphala, gallic acid, chebulinic acid, Phyllanthus emblica, Terminalia bellirica, Terminalia chebula*

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1. Introduction

Triphala is a plant product which has a long history of usage in India. Triphala is composed of *P. emblica*, *T. bellerica* and *T. chebula* usually in the 1:1:1 composition [1]. It is believed that regular consumption of Triphala leads to good health and longevity. There have been reports on the biological activities of Triphala including anti-cancer, anti-inflammatory, anti-diabetic, muscle-relaxing, anti-microbial, anti-depression, immune-enhancing and ulcer-healing activities [2].

The main constituents of Triphala products were found to be gallic acid, ellagic acid, chebulinic acid, ascorbic acid, syringic acid, tannic acid and other phenolic compounds [1,3]. In Thailand Triphala products enjoy increasing popularity and there are a number of formulations (with sugar and sugar-free) with different proportions of the three plants. There have been reports that four formulations of Triphala are effective in enhancing the immune system. It has been suggested that formulations with equal proportion of the three plants are recommended for daily consumption while those with higher proportions of *T. bellerica* are suitable for relieving allergic symptoms. There were reports on the uric acid-lowering, anti-inflammability, blood pressure-reducing and blood lipid-lowering activities. For Triphala products to be effective, the exact composition of the three plants is of vital importance [4,5,6]. To date, there have been no studies on the seasonal variation of the active constituents of the three plants, leading to uncertain composition of the Triphala products.

Therefore, it was decided to investigate the seasonal variation of the active constituents of the three plants (collected in 2017 and 2018 seasons) by studying the gallic acid and chebulinic acid contents of the hexane, ethyl acetate and methanol extracts. The gallic acid and chebulinic acid (Figure 1) contents in the methanol extract of Triphala products (2:1:1; with sugar and sugar-free) for 2017 and 2018 were also studied to establish the correlation of the contents of these two acids in the plants

and in the Triphala products in the same period. It was anticipated that the study will be useful for the future production of Triphala products with desired compositions of the two acids.

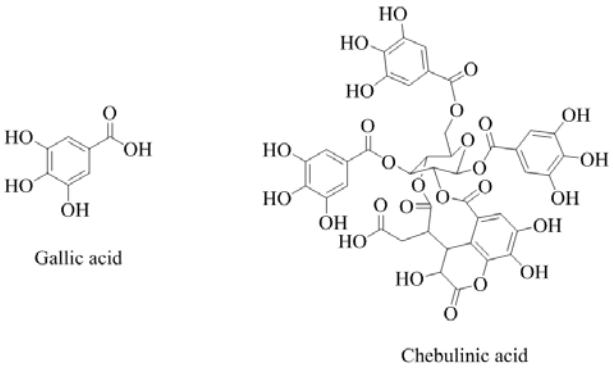

Figure 1. Structure of Gallic and Chebulinic acid

2. Materials and Methods

2.1. Plant Material

P. emblica (BKF 113645), *T. bellirica* (BKF 188656) and *T. chebula* (BKF 170476) were collected from Chae Son National Park (18°45′12″N, 99°24′32″E.) , Lampang, Thailand in January, 2017 and 2018 by Mr. Narong Nuntasaen. The plants were identified and deposited in the Forest Herbarium, Department of National Park, Wildlife and Plant Conservation, Ministry of National Resources and Environment, Bangkok, Thailand**.** Gallic acid monohydrate and chebulinic acid were purchased from Sigma-Aldrich Co.

2.2. Plant Extraction

The dried fruits (collected in 2017 and 2018) (2 kg) of *P. emblica*, *T. bellirica* and *T. chebula* (Figure 2) [7] were ground into powder and then separately successively extracted with hexane, ethyl acetate and methanol (5×7 L for each solvent). Removal of the solvents from each extract under reduced pressure gave the crude extracts of *P. emblica*, *T. bellirica* and *T. chebula.* Dry Triphala (2017 and 2018) in 2:1:1 (*P. emblica*, *T. bellirica* and *T. chebula*) proportions was extracted only with methanol to give the crude methanol extract.

Figure 2. Dry Fruits of *P. emblica*, *T. bellirica* and *T. chebula*

2.3. The Crude Extract Preparation

The weights of all extracts were 20.0-21.0 ± 0.9 mg. They were separately extracted with methanol (1 mL) under vortex mixture for 3 minutes, and filtered through nylon membrane. The filtrates were stored under 25 °C and the gallic acid and chebulinic acid contents were determined using HPLC.

2.4 Type of Triphala Drink on 2017 and 2018 Years

The two main types of Triphala drinks available in the market are sugar and without sugar which are totally different in the ratio of ingredients. The type of Triphala drink on 2017 and 2018 years are shown in Table 1.

Table 1. Type of Triphala drink on year 2017 and 2018

Ingredient	Triphala with sugar drink (100 mL)	Triphala without sugar drink (100 mL)
Water	87.00 mL	81.01 mL
P. emblica	1.50 g	8.86 g
T. bellirica	1.30 g	5.06 g
T. Chebula	1.30 g	5.06 g
Salt (NaCl)	0.05 g	0.01 g
Sugar	8.70 g	-

2.4. HPLC Analysis

HPLC analysis was carried out on a HITACHI Chromaster equipped with a 5110 pump, a 5310 column oven, a 5430 diode array detector and a 5210 auto sampler. Separation was undertaken on a HITACHI LaChrom C_{18} column (250 × 4.6 mm, 5 µm) at 25 °C. The mobile phase flow rate was 1 mL/min. The injection volume was 10 µL. The quantitation wavelength was set at 270 nm. Identification of gallic acid and chebulinic acid was accomplished by comparing the retention times and absorption spectra of relevant peaks to those of standard compounds. The separation condition for HPLC analysis was modified according to previous papers [8]. The mobile phase consisted of 1% acetic acid in water (A), pH 2.65 and methanol (B). The program for gradient elution started at 90% solvent A and 10% solvent B, linearly increased to 50% solvent A and 50% solvent B in 25 min, followed by washing for 25 min (Table 2).

Table 2. The gradient system used in chromatographic separation

Step gradient		
Time (min)	1% Acetic acid in water (%V/V)	Methanol (%V/V)
0	90	10
10	80	20
15	72	28
20	65	35
25	50	50
26	0	100
30	0	100
32	90	10
40	90	10
50	90	10

2.5. Gallic Acid and Chebulinic Acid Measurement

Quantification estimation of gallic acid and chebulinic acid were carried out based on the calibration curves of standard gallic acid and chebulinic acid. Solutions of 15.625, 31.25, 62.5, 125, 250, 500 and 1000 µg/mL of gallic acid and chebulinic acid were prepared. The standard curves were plotted between the peak areas and concentrations. Peak areas of gallic acid monohydrate were compared to the standard curve and calculated to give their contents. The solutions were filtered through a nylon membrane (0.45 mm) syringe filter and analyses were carried out in triplicate. Peak area of standard gallic acid and chibulinic acid are shown in Table 3 and Table 4.

3. Results and Discussion

3.1. Extraction Yield

The total weights of the relevant extracts are shown in the Table 5.

From Table 5, it could be concluded that the weights of the extracts increased with increasing polarity of the solvents. This is consistent with previous reports of the occurrence of ascorbic acid, gallic acid, amalic acid, chebulic acid, chebulinic acid and quercitin in *P.emblica* [9] *T. bellirica* fruit was found to contain ellargic acid, gallic acid, chebulaginic acid, bellericanin and chebulinic acid [10] while *T. chebula* fruit was found to contain gallic acid, tannin, chebulic acid and chebulinic acid [11]. According to solvent extraction, researches on Triphala indicated that the methanol extracted reviewed the highest bioactivities and the higher amount of phenolic compounds including gallic and chebulinic acid than the others. Then, only Triphala (2:1:1) was extracted by methanol. [12,13,14,15]

3.2. Calibration Curve

Gallic acid and chebulinic acid standards were used as references to construct the standard curves for the calculation of sensitivity, stability, precision, repeatability, and recovery rate; the method was validated in terms of all these parameters. The parameters related to the standard curves, such as regression equation, correlation coefficient, and linear range are shown in Table 6. The calibration curves of gallic acid and chebulinic acid showed good linearity ($R^2 \geq 0.9998$) within the test range, and their content could be accurately determined using the regression equation. (Figure 3 and Figure 4)

Table 3. Peak area of standard gallic acid from HPLC chromatogram

[Gallic acid], µg/mL	Area, AU-min	Rep1	Rep2	Rep3	Mean	SD
15.625	0	0	0	0	0	0
31.25	733965.75	12173644	11945952	11938753	12019449.70	133584.71
50	1206632.33	6182932	6140792	6104102	6142608.67	39446.39
62.5	1497574.33	3102408	3077936	3102106	3090941	13139.74
125	3090941.00	1504922	1496590	1491211	1497574.33	6908.30
250	6142608.67	1234044	1201020	1184833	1206632.33	25080.96
500	12019449.67	735875	733653	738163	733965.75	4278.92

Au-min means absorbance unit-minute.

Table 4. Peak area of standard chibulinic acid from HPLC chromatogram

[Chebulinic acid], µg/mL	Area, AU-min	Rep1	Rep2	Rep3	Mean	SD
31.25	61649.33	62809	61337	60802	61649.33	1039.32
62.5	150708.70	150659	150786	150681	150708.70	67.87
125	319171.30	315677	317285	324552	319171.30	4728.64
250	656751.00	657586	656688	655979	656751.00	805.35
500	1318594.00	1277923	1329122	1348738	1318594.00	36562.48

Au-min means absorbance unit-minute.

Table 5. Yield of all crude extract of Triphala plant on 2017 and 2018

Plants	Total weight of raw material (g)	Solvent	2017		2018	
			Total weight of crude extract (g)	% yield	Total weight of crude extract (g)	% yield
P. emblica	2000	Hexane	18.70	0.94	33.55	1.68
	2000	EtOAc	106.92	5.35	72.04	3.60
	2000	Methanol	465.66	23.28	622.02	31.10
T. bellirica	2000	Hexane	24.80	1.24	35.42	1.77
	2000	EtOAc	73.96	3.70	63.83	3.19
	2000	Methanol	562.19	28.11	571.43	28.58
T. chebula	2000	Hexane	24.68	1.23	24.00	1.20
	2000	EtOAc	112.69	5.63	61.00	3.05
	2000	Methanol	676.37	33.82	1038.00	51.90
Triphala (2:1:1)	2000	Methanol	1016.52	50.83	1200.00	60.00

Table 6. Standard curves, the LOD and LOQ of gallic acid and chebulinic acid contents

Standards	Regression equation (µg/mL)	Correlation coefficient (R^2)	LOD (µg/mL)	LOQ (µg/mL)
Gallic acid	y = 20495x + 24030	0.9998	0.54	1.08
Chebulinic acid	y = 2672.1x − 17319	0.9999	1.88	6.22

LOD means limit of detection
LOQ means limit of quantitation.

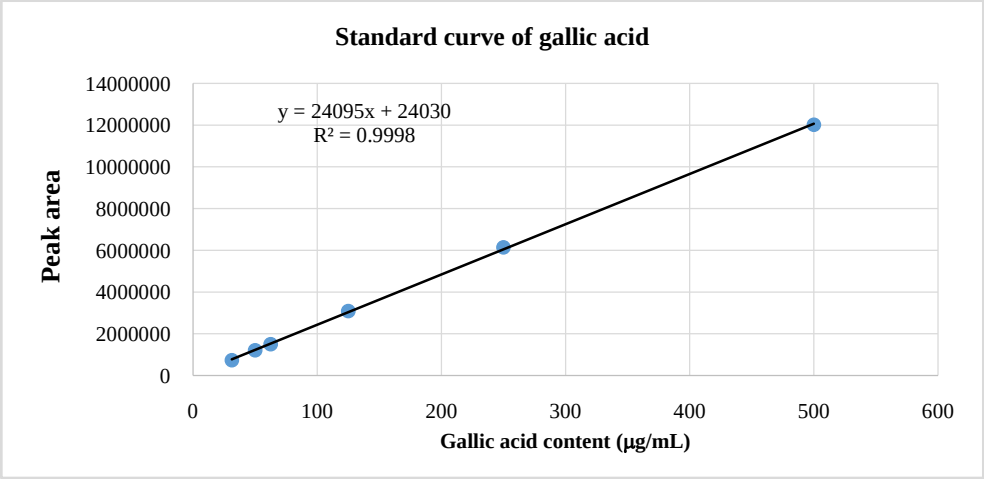

Figure 3. Standard curve of gallic acid

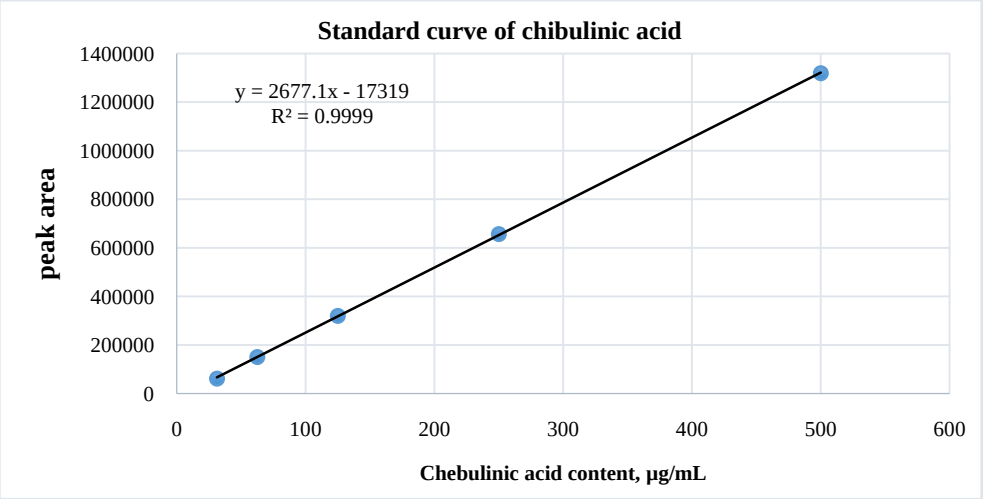

Figure 4. Standard curve of chebulinic acid

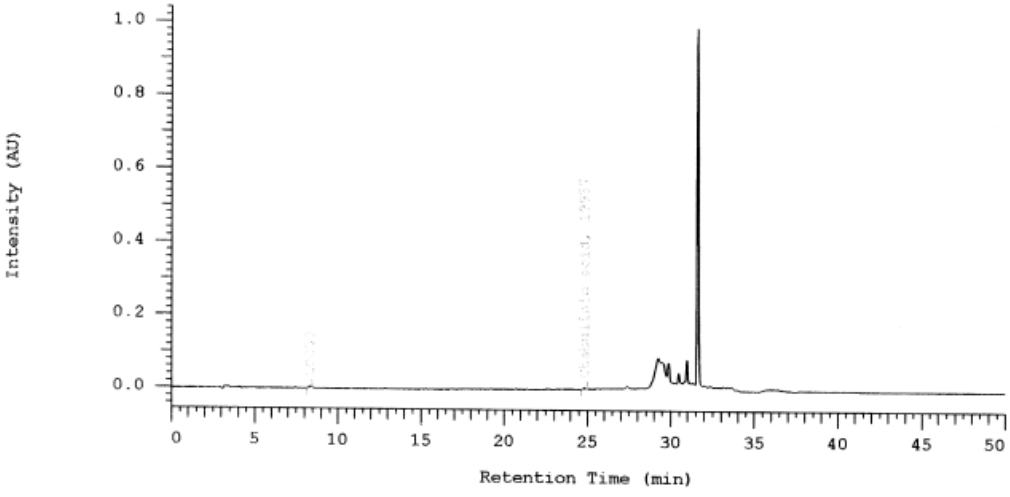

Figure 5. HPLC chromatograms of *P.emblica*-Hexane extract-2017 at 270 nm

3.3. HPLC Chromatogram

Retention time values of gallic acid and chebulinic acid were found to be 7.56 and 22.8 minutes respectively, consistent with previous reports [8] (Figure 5). HPLC chromatogram of *P. emblica*, *T. bellirica*, *T. chebula*, Triphala(2:1:1), Triphala herbal drink (sugar-free) and Triphala herbal drink (with sugar) in 2017 and 2018 were shown in Figure 5– Figure 15 and 16-27. (Chromatogram which appear after the 26th minute would not be considered, were column washing period.)

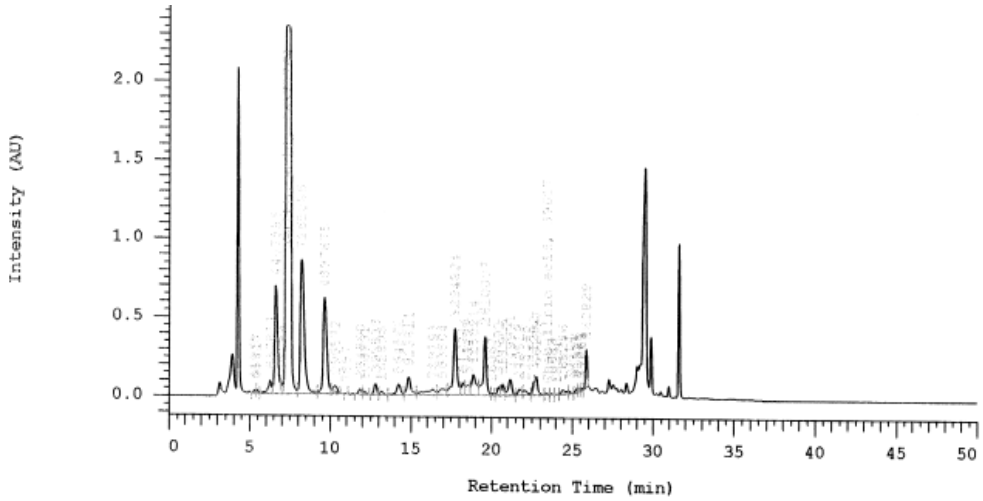

Figure 6. HPLC chromatograms of *P.emblica*-EtOAc extract-2017 at 270 nm

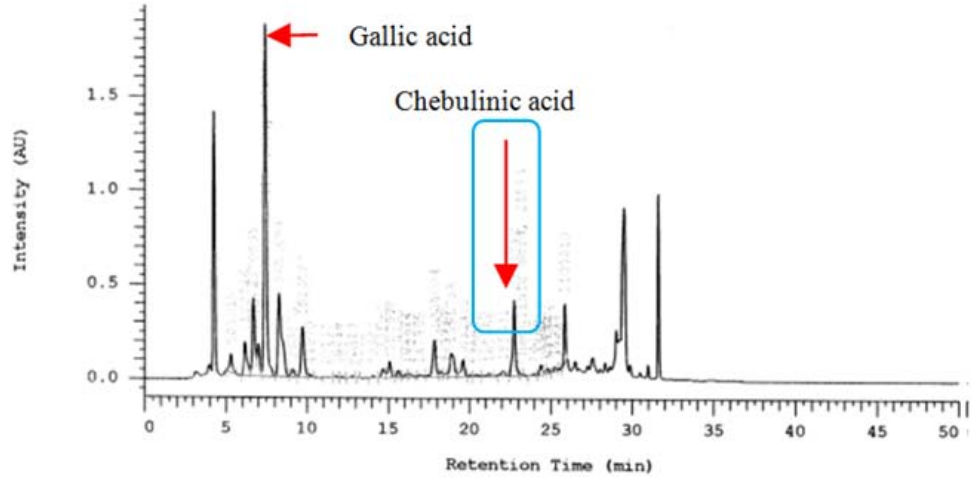

Figure 7. HPLC chromatograms of *P.emblica*-MeOH extract-2017 at 270 nm

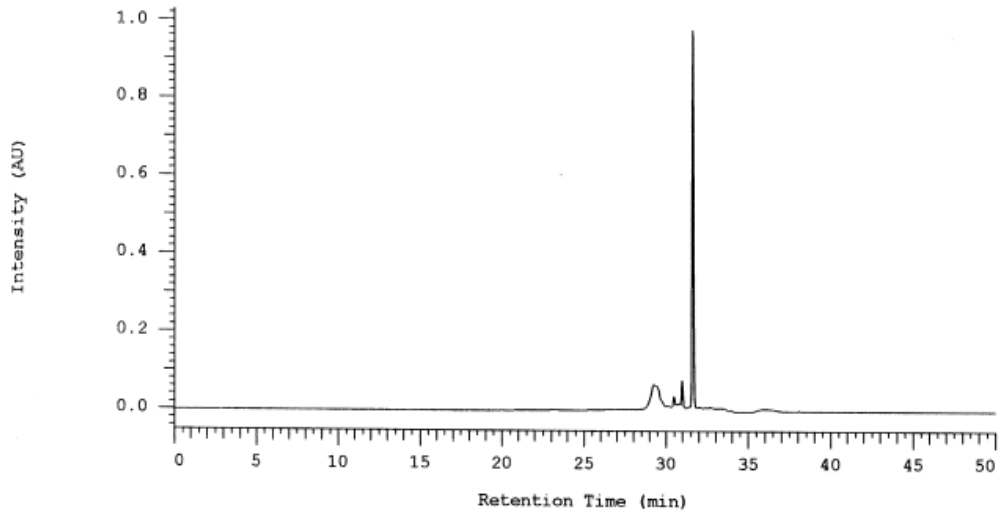

Figure 8. HPLC chromatograms of *T. bellirica* -Hexane extract-2017 at 270 nm

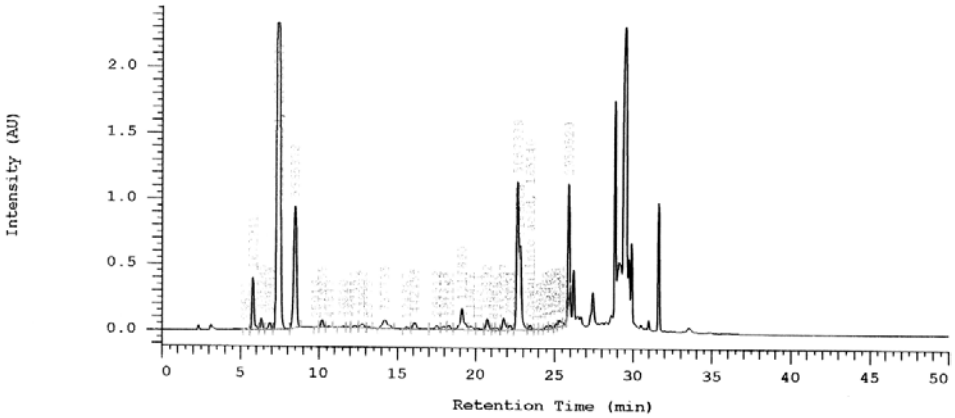

Figure 9. HPLC chromatograms of *T. bellirica* -EtOAc extract-2017 at 270 nm

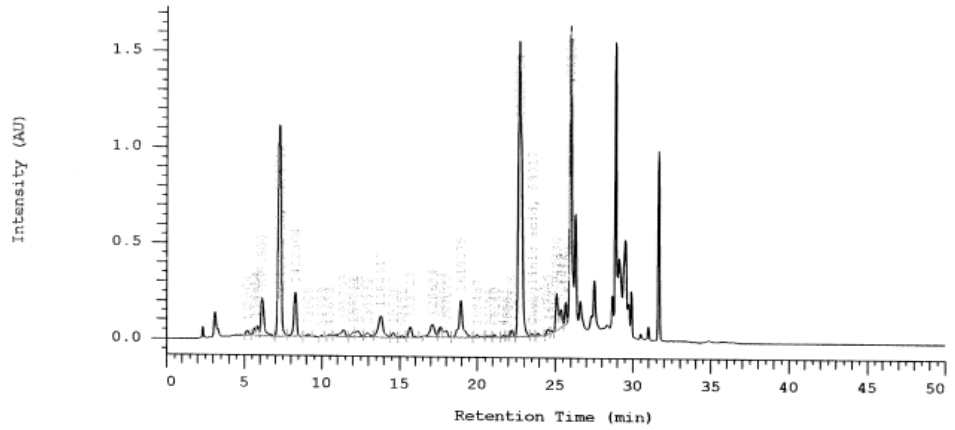

Figure 10. HPLC chromatograms of *T. bellirica* -MeOH extract-2017 at 270 nm

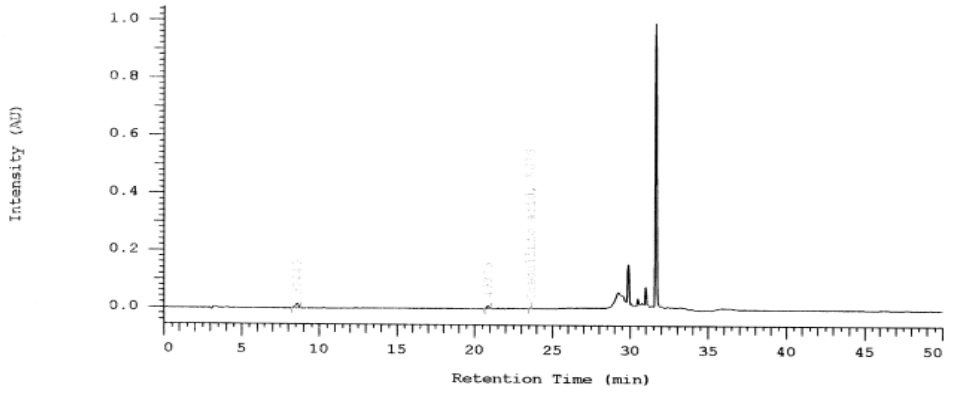

Figure 11. HPLC chromatograms of *T. chebula* -Hexane extract-2017 at 270 nm

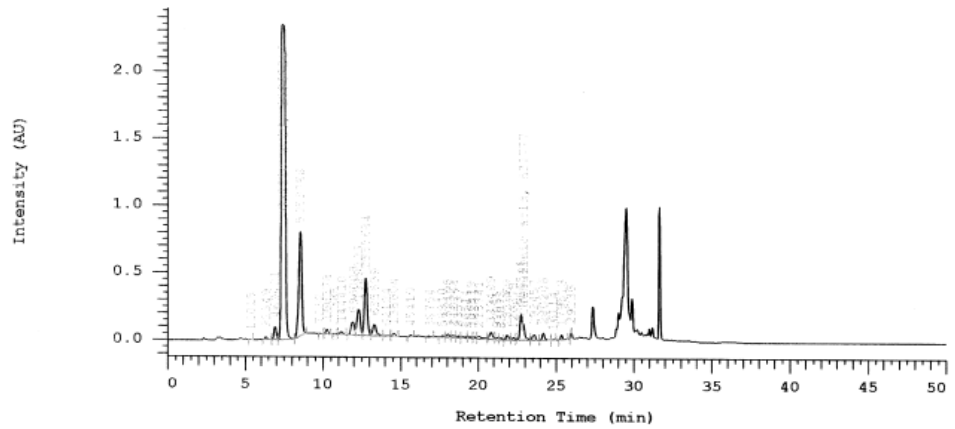

Figure 12. HPLC chromatograms of *T. chebula* -EtOAc extract-2017 at 270 nm

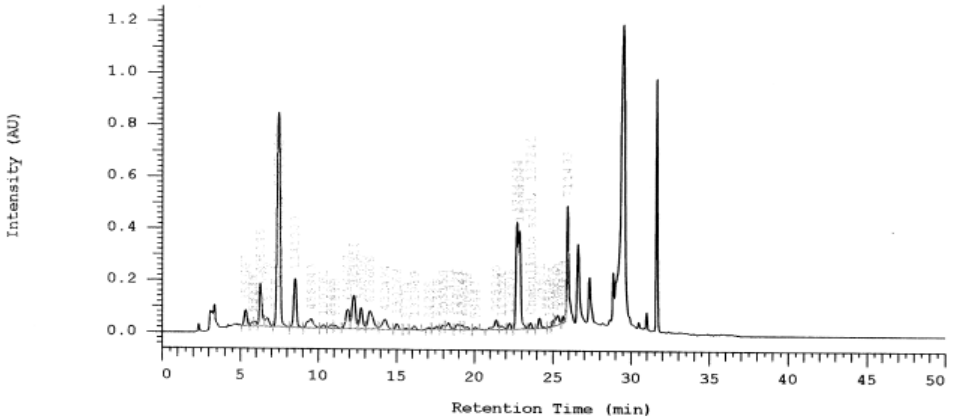

Figure 13. HPLC chromatograms of *T. chebula* -MeOH extract-2017 at 270 nm

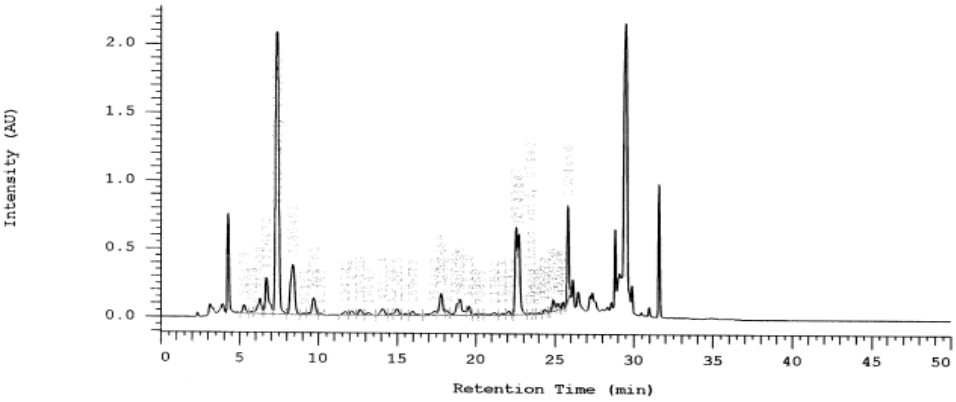

Figure 14. HPLC chromatograms of Triphala (2:1:1)-MeOH-2017 at 270 nm

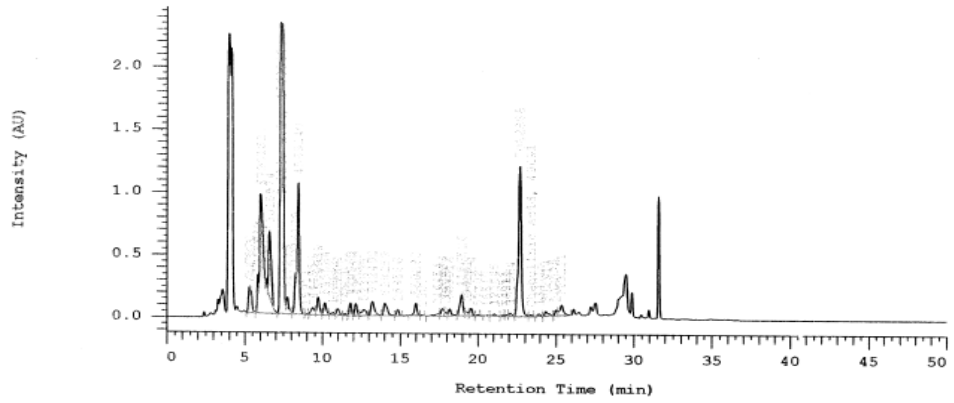

Figure 15. HPLC chromatograms of Triphala herbal drink (sugar-free) on year 2017 at 270 nm

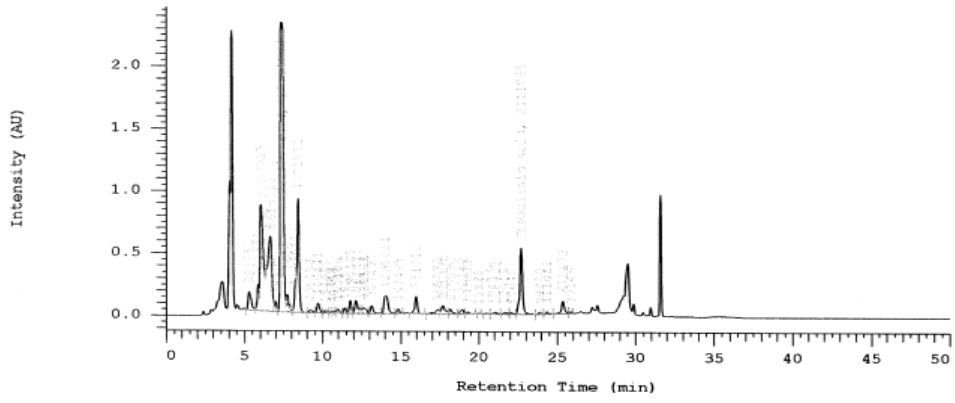

Figure 16. HPLC chromatograms of HPLC chromatogram of Triphala herbal drink (with sugar) on year 2017 at 270 nm

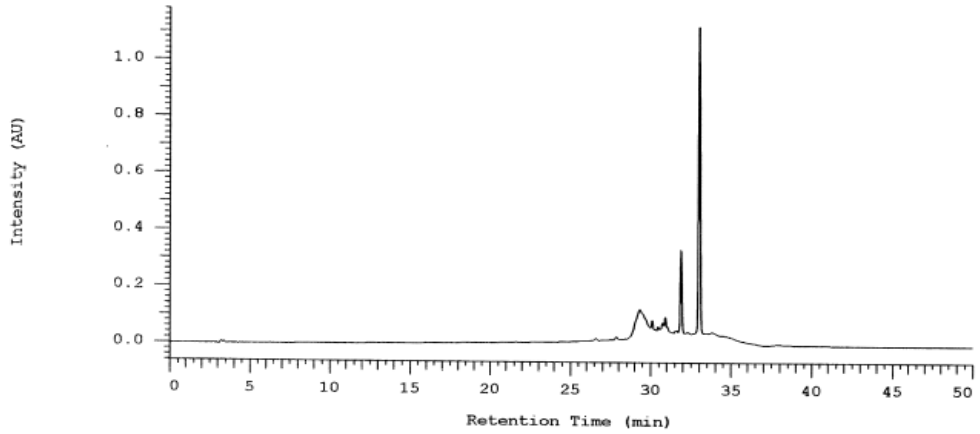

Figure 17. HPLC chromatograms of *P.emblica*-Hexane extract-2018 at 270 nm

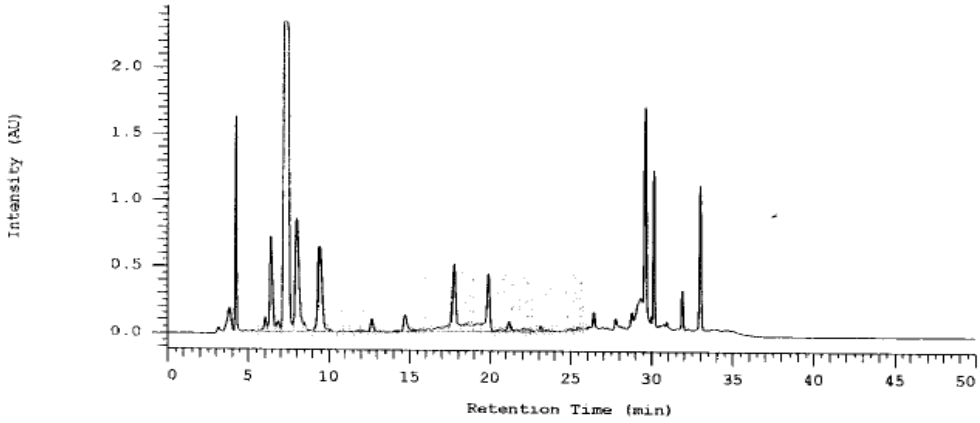

Figure 18. HPLC chromatograms of *P.emblica*-EtOAc extract-2018 at 270 nm

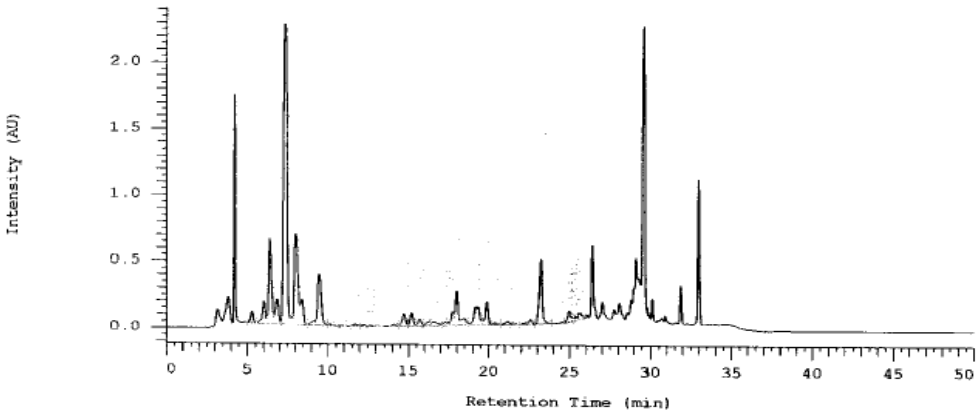

Figure 19. HPLC chromatograms of *P.emblica*-Methanol extract-2018 at 270 nm

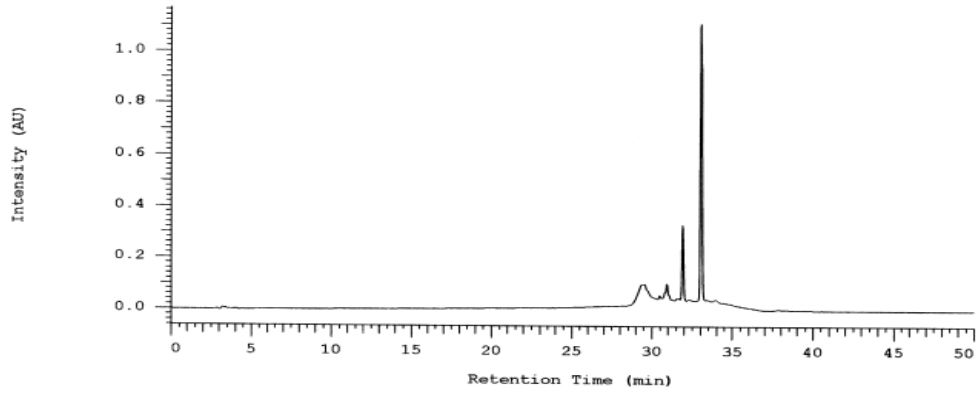

Figure 20. HPLC chromatograms of *T. bellirica* -Hexane extract-2018 at 270 nm

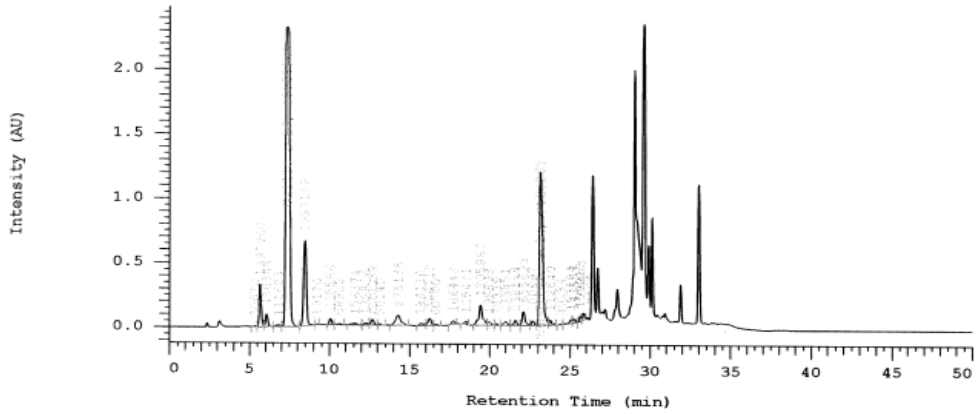

Figure 21. HPLC chromatograms of *T. bellirica* -EtOAc extract-2018 at 270 nm

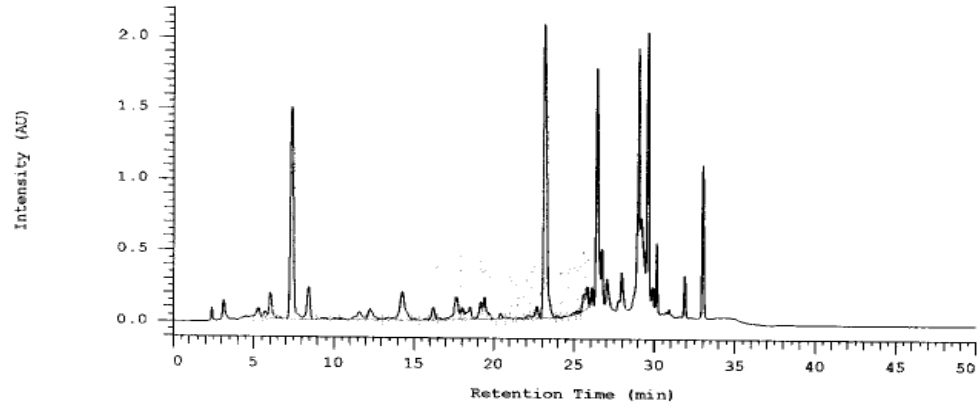

Figure 22. HPLC chromatograms of *T. bellirica* -MeOH extract-2018 at 270 nm

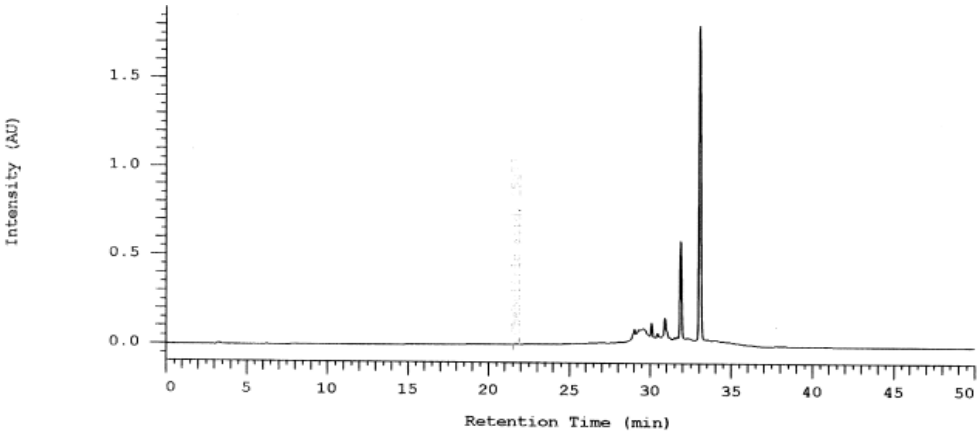

Figure 23. HPLC chromatograms of *T. chebula* -Hexane extract-2018 at 270 nm

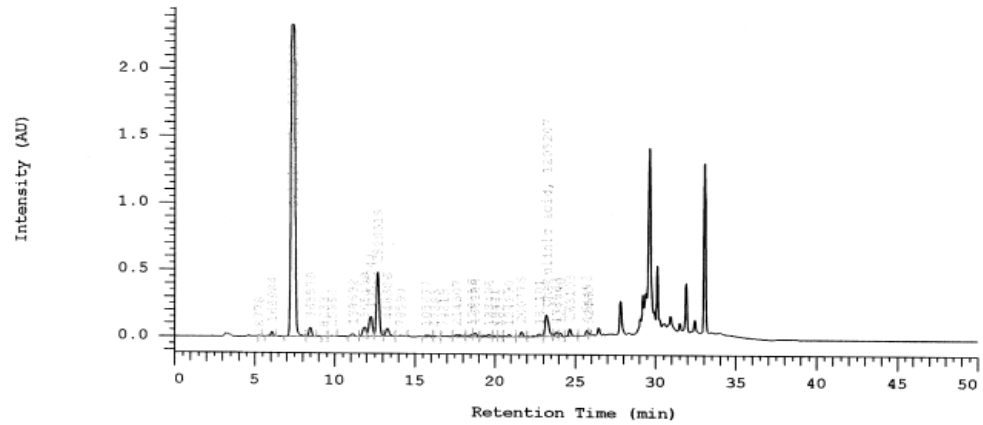

Figure 24. HPLC chromatograms of *T. chebula* -EtOAc extract-2018 at 270 nm

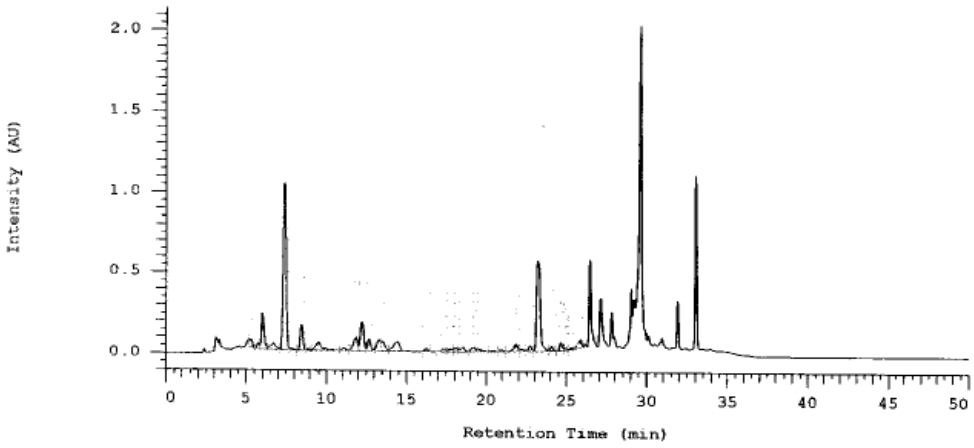

Figure 25. HPLC chromatograms of *T. chebula* -MeOH extract-2018 at 270 nm

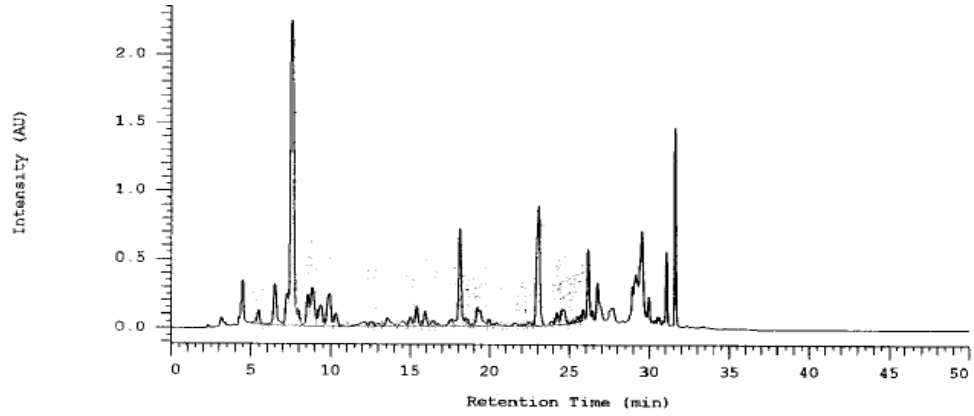

Figure 26. HPLC chromatograms of Triphala (2:1:1)-MeOH-2018 at 270 nm

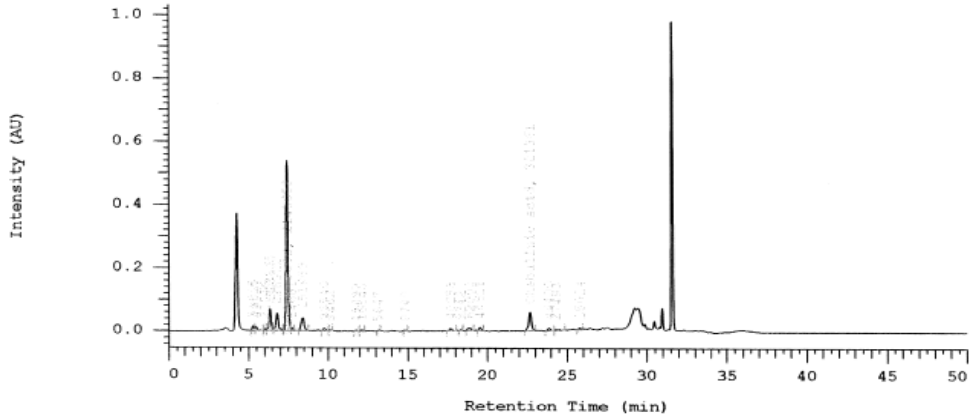

Figure 27. HPLC chromatograms of Triphala herbal drink (sugar-free) on year 2018 at 270 nm

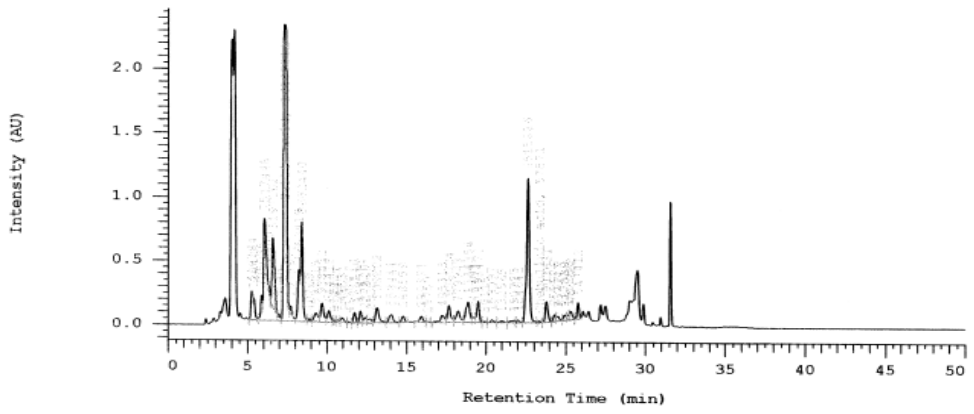

Figure 28. HPLC chromatograms of HPLC chromatogram of Triphala herbal drink (with sugar) on year 2018 at 270 nm

3.4. Analysis of Gallic Acid and Chebulinic Contents in Triphala Herb Samples

The contents of gallic acid, chebulinic acid in different extracts of Triphala herb and Triphala herbal drinks are listed in Table 7, Table 8, Table 9 and Table 10.

Table 7. Gallic acid contents of different extracts of the three Triphala herbs (collected in 2017 and 2018 years)(g/100 g dry weight)

Herb	Extract	Year 2017	Year 2018
P. emblica	Hexane	ND	ND
	EtOAc	0.28^{a}	0.17^{b}
	MeOH	0.46^{b}	1.06^{a}
T. bellirica	Hexane	ND	ND
	EtOAc	0.15^{a}	0.12^{b}
	MeOH	0.40^{b}	0.57^{a}
T. chebula	Hexane	ND	ND
	EtOAc	0.23^{a}	0.13^{b}
	MeOH	0.36^{b}	0.70^{a}
Triphala (2:1:1)	MeOH	1.47^{b}	2.05^{a}

*Each value is expressed as mean SD (n=3)
Mean followed by different letters within a row are significantly different ($p < 0.05$)
ND is not detected.

The crude methanol extracts of the three plants possessed the highest contents of gallic acid, followed by the ethyl acetate extracts while gallic acid was not found in the hexane extracts. The contents of gallic acid in the three herbs ranged from 0.15 g/100 g in the ethyl acetate extracts of *T. bellirica* to 0.46 g/100 g in the methanol extracts of *P. emblica* for 2017. For 2018, the gallic acid contents in the methanol extracts ranged from 1.06 g/100 g for *P. emblica* to 0.12 g/100 g for *T. bellirica*. In the case of Triphala (2: 1: 1) products, the gallic acid content for 2018 was found to be 28% higher than that for 2017.

Table 8. Gallic acid content of Triphala herbal drink on year 2017 and 2018 (g/100mL)

Triphala herbal drinks	Year 2017	Year 2018
Triphala with sugar-free^{ns}	3.64	3.61
Triphala with sugar	4.26^{a}	3.56^{b}

ns means non significant.

The results of the determination of gallic acid content in both formulas in 2017 and 2018 showed that the amount of gallic acid ranged from 3.56-4.26 g /100 mL. The contents of chebulinic acid in different extracts and herb samples are listed in Table 9.

Table 9. Chebulinic acid content of Triphala herb at different solvent extract on year 2017 and 2018 (g/100 g dry weight)

Plant	Extract	Year 2017	Year 2018
P. emblica	Hexane	ND	ND
	EtOAc	0.06^{a}	0.01^{b}
	MeOH	0.94^{b}	1.71^{a}
T. bellirica	Hexane	ND	ND
	EtOAc	0.34^{b}	0.42^{a}
	MeOH	5.83^{b}	7.71^{a}
T. chebula	Hexane	ND	ND
	EtOAc	0.09^{a}	0.01^{b}
	MeOH	1.20^{b}	4.37^{a}
Triphala (2:1:1)	MeOH	2.08^{b}	7.21^{a}

*Each value is expressed as mean SD (n=3).
Mean followed by different letters within a row are significantly different ($p < 0.05$)
ND is not detected.

In the methanol extracts, the contents of chebulinic acid in the three herbs ranged from 0.06 g/100 g for *P. emblica* to 5.83 g/100 g for *T. bellirica*. For 2017, the contents of chebulinic acid in other extracts for the 2 years did not show a clear trend. The content of chebulinic acid in both Triphala formulas are shown in the Table 10.

Table 10. Chebulinic acid contents of Triphala herbal drinks on for 2017 and 2018 years (g/100 mL dry weight)

Triphala herbal drink	Year 2017	Year 2018
Triphala with sugar-free	0.26^{b}	0.58^{a}
Triphala with sugar	0.10^{b}	0.60^{a}

*Each value is expressed as mean SD (n=3)
Mean followed by different letters within a row are significantly different ($p < 0.05$)
ND is not detected.

The chebulinic acid contents in both formulas for 2017 and 2018 ranged from 0.10-0.60 g /100 mL. In *P. emblica*, the contents of gallic acid were 0.17-1.06% and those of chebulinic acid were 0.01-1.71%. In *T. bellirica*. The gallic acid contents were 0.12-0.57% and those of chebulinic acid were 0.34-7.71%. In *T. chebula*, the gallic acid contents were 0.13-0.70% and those of chebulinic acid were 0.01-4.37%. In Triphala (2:1:1) the gallic acid contents were 1.47-2.05% and those of chebulinic acid were 2.08-7.21%. In sugar-free Triphala herbal drinks, the gallic acid contents were 3.61-3.64% and those of chebulinic acid were 0.10-0.26%. In Triphala with sugar herbal drinks, the gallic acid contents were 3.56-4.26% and those of chebulinic acid were 0.58-0.60%.

4. Conclusion

Determination of the gallic acid and chebulinic acid contents of *P. emblica*, *T. bellirica* and *T. bellirica* as well as Triphala products for 2017 and 2018 revealed that there were seasonal variations of these constituents which may have a significant impact on the quality of these Triphala products. In order to offer a consistent and desirable quality in these Triphala products, determination of the gallic acid and chebulinic acid contents prior to production is an important necessity.

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