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HPLC-MS PROFILES AND QUANTITATIVE ANALYSIS OF TRIPHALA FORMULATION

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Abstract: Triphala, a polyherbal formulation in Ayuraveda, is composed of *Terminalia chebula* Retz., *Terminalia bellirica* Roxb., and *Phyllanthus emblica* Linn fruits. This formulation has been prescribed for chronic constipation, colon detoxification, and body rejuvenation. Standardization of Triphala formulation has been established to the quantitative determination of gallic acid. This study aimed to develop HPLC analytical method for determination of Triphala and modified Triphala formulations comparing with the individual herbs. HPLC analysis was performed on a C18 column and 1%acetic acid in water and acetonitrile in step gradient as the mobile phase. The peak areas were recorded at the wavelengths of 270 nm for gallic acid and rutin using photodiode array detector. HPLC profiles of Triphala and modified Triphala formulations were in similar patterns but relatively different from those of the individual herbs. Electrospray mass spectrometry (ESI-MS) was used to identify the chemical compounds found in Triphala formulations. The results showed that ascorbic acid, gallic acid, corilagin, chebulagic acid and chebulinic acid were eluted in order. The gallic acid contents of Triphala and modified Triphala formulations were in the range of 2.66-2.87 %w/w comparing with 1.32-4.02%w/w of the individual herbs, while their rutin contents were in the range of 0.65-2.94 %w/w comparing with 0.61-2.61%w/w of the individual herbs.

Keywords: ESI-MS, Gallic acid, HPLC, Rutin, Triphala

บทคัดย่อ: ตรีผลาเป็นตำรับยาสูตรผสมใช้ในอายุรเวทประกอบด้วยผลของสมุนไพร 3 ชนิด คือ สมอไทย สมอพิเภก และมะขามป้อม ตำรับยานี้นำมาใช้ในการรักษาอาการท้องผูกเรื้อรัง ขจัดพิษในลำไส้ และทำให้ร่างกายกระปรี้กระเปร่า การควบคุมมาตรฐานของตำรับตรีผลาใช้การวิเคราะห์เชิงปริมาณของกรดแกลลิก การศึกษาครั้งนี้มีจุดมุ่งหมายเพื่อพัฒนาวิธีวิเคราะห์ตรีผลาด้วยเทคนิคเฮชพีแอลซี เพื่อใช้ในการวิเคราะห์ตำรับตรีผลาและตรีผลาสูตรปรับปรุงเปรียบเทียบกับสมุนไพรเดี่ยวที่เป็นส่วนประกอบ การวิเคราะห์ด้วยเฮชพีแอลซีใช้คอลัมน์ C18 และใช้กรดอะซิติกความเข้มข้น 1% ในน้ำ และอะซิโตไนไตร์เป็นเฟสเคลื่อนที่แบบ step gradient บันทึกค่าพื้นที่ใต้พีกที่ความยาวคลื่น 270 นาโนเมตรสำหรับกรดแกลลิกและรูติน ด้วยระบบตรวจวัดสัญญาณแบบโฟโตไดโอดอะเร รูปแบบโครมาโตแกรมของตำรับตรีผลาและตรีผลาสูตรปรับปรุงมีลักษณะการแยกคล้ายกันแต่แตกต่างจากรูปแบบการแยกของสมุนไพรเดี่ยวแต่ละชนิดที่เป็นส่วนประกอบ เทคนิคอิเล็กโตรสเปรย์แมสสเปกโทรเมทรีนำมาใช้ในการพิสูจน์เอกลักษณ์ของสารที่เป็นองค์ประกอบในตำรับตรีผลา ผลการศึกษาพบกรดแอสคอร์บิก กรดแกลลิก คอริลาจิน กรด เชบูลาจิก และกรดเชบูลินิก แยกออกมาตามลำดับ ปริมาณของกรดแกลลิกในตำรับตรีผลาและตรีผลาสูตรปรับปรุงอยู่ในช่วงร้อยละ 2.66-2.87 โดยน้ำหนัก เทียบกับร้อยละ 1.32-4.02 โดยน้ำหนัก ในสมุนไพรเดี่ยวในขณะที่ปริมาณรูตินอยู่ในช่วงร้อยละ 0.65-2.94 โดยน้ำหนักเทียบกับร้อยละ 0.61-2.61 โดยน้ำหนักของสมุนไพรเดี่ยว

คำสำคัญ: อิเล็กโตรสเปรย์แมสสเปกโทรเมทรี, กรดแกลลิก, เฮชพีแอลซี, รูติน, ตรีผลา

INTRODUCTION

It is well known that Triphala formulation has been used in Ayuraveda for a long time. It has been prescribed for chronic constipation, colon detoxification, and body rejuvenation. It can cause diarrhea as a minor side effect. In Thailand it has been official in the list of herbal medicinal product A.D. 2012 in tablet, pill, capsule and powder dosage forms (Department for Development of Thai Traditional and Alternative Medicine, 2012). It is indicated to use for some respiratory symptoms especially relieving cough. Triphala composes of dried fruits of three herbal plants; *Terminalia bellirica* Roxb., *Terminalia chebula* Retz., and *Phyllanthus emblica* Linn. These plants have been used for laxative, carminative, astringent and expectorant. Chemical compounds which are found in these plants are benzenoids, flavonoids, tannins and coumarin such as gallic acid, chebulinic acid, chebulagic acid, hydrolysable tannins, quercetin and rutin. The contents of these compounds are different in the formulation due to the quality of raw material, the method of preparation and the proportion of each plant in the formulation.

Triphala formulation usually is prepared in the ratio 1 : 1 : 1 of those three plants. However, Thai traditional medicine is prepared in different ratios based on the symptoms of patients and hypothesis of diseases. Pita, Watta and Semha formula of Triphala compose of *T. bellirica* Roxb., *T. chebula* Retz., and *P. emblica* Linn. in the ratios of 12:8:4; 4:12:8 and 8:4:12, respectively (Chaowalitthumrong, 1997). Pita formula of Triphala was used to alleviate the fire element in Summer, Wata was used to relieve wind element in Summer and Semha was used to remove water element in Summer. There was a subacute toxicity study of Triphala water extract in Wistar rats. That article reported the administration of 0.36, 2.88 and 23.4 g of the crude extract/kg body weight/day reduced the body weight and food consumption comparing to the control group. Wata extract had lowered white blood cell numbers while Pitta and Semha did not changed any hematological parameters of the rats and it did not correlated with the dose of the extracts. In addition, biochemical studies of serum samples showed that high doses of the extracts significantly decreased total plasma protein and BUN levels in male rats. Hepatotoxic and nephrotoxic effects of Triphala formula may due to the high tannin content. Therefore, it is interesting to determine the contents of major compounds found in Triphala formulations.

There are some research studies reporting about spectrophotometric and chromatographic methods for analysis and identification the chemical contents in Triphala. There was a study reporting the content of rutin and gallic acid in Triphala curna were 0.67±0.005 and 0.76±0.01 µg/ml analyzing by spectrophotometric method (Pawar & Salunkhe, 2013). There was HPTLC method determining ellagic and gallic acid contents in polyherbal formulation which contained *P. emblica* as the major ingredient (Syam Vardhan, 2012). Several papers reported HPLC analytical methods for determining Triphala (Pawar, 2009; Patel Madhavi, 2010; Mahajan, 2011). Another article reported a validated HPLC method for determining chemical constituents in four Terminalia species (Dhanani, 2014). Reversed phase chromatography was optimized and selected for this study. MS/MS was used to confirm the molecular weight of the main constituents in Triphala.

MATERIALS AND METHODS

Materials

Gallic acid (CAS No. 149-91-7) and rutin hydrate (CAS No. 5995-86-8) were purchased from Sigma-Aldrich (USA). Methanol and acetonitrile (HPLC grade) were purchased from Burdick&Jackson Honeywell (Korea). Other chemicals were analytical grade obtained from Univar (USA).

Plant preparation and extraction

The whole dried fruits of *T. chebula*, *T. bellirica*, and *P. emblica* were purchased from Charoensuk traditional manufacturing, Nakorn Pathom province and ground to powder, then, prepared in the ratio according to Table 1. The total weight of each formula/herbal plant was 8.4 g. The powders were boiled in 840 ml boiled water for 15 min, let them cool down, centrifuged at 5,000 rpm for 10 minutes, and filtered through Whatman no.1 paper. The filtrate was freeze dried using lyophilizer. The aqueous extracts were stored in glass bottle, kept in desiccator and protected from light. The aqueous extract of each formula was determined the main components using HPLC.

Table 1. The proportion of herbal plants in Triphala and modified Triphala formula

Formula/herbal plant	Proportion of each herbal plant			Note
	T. chebula	*T. bellirica*	*P. emblica*	
T.F.* 1	1	1	1	List of Herbal Medicinal Products A.D.2012
MT.F.** 2	2	3	1	Pitta formula
MT.F.** 3	3	1	2	Wata formula
MT.F.** 4	1	2	3	Semha formula
MT.F.** 5	1	2	4	
T. chebula	1	-	-	
T. bellirica	-	1	-	
P. emblica	-	-	1	

* T.F. = Triphala formula

** MT.F. = Modified Triphala formula

HPLC method development for Triphala determination

HPLC analysis was performed on an Agilent 1260 series equipped with photodiode array detector and autosampler. Data analysis was carried out using OpenLab CDS EZChrome software (Agilent, USA). Separation was performed on an ACE Generix C18 column (250×4.6 mm, 5 µm) at the temperature of 25 °C. The mobile phase flow rate was 1 mL/min. The injection volume was 10 µL. The quantitation wavelength was set at 270 and 360 nm. The mobile phase consisted of 1%acetic acid in water, pH 2.65 (A) and acetonitrile or methanol (B). The development was modified on the mobile phase: 1) linear gradient 0-100% methanol in 20 min 2) linear gradient 0-100% acetonitrile in 20 min 3) step gradient 10-50% acetonitrile in 25 min, and 4) step gradient 5-50% acetonitrile in 35 min. The chromatographic separation was achieved using a step gradient program as shown in Table 2.

Electrospray mass spectrometry condition

ESI MS was performed on a Bruker Amazon SL mass spectrometer coupling with HPLC Ultimate 3000, Dionex (US). Poroshell 120 C18 column was used as a stationary phase and the mobile phase composition was 1%acetic acid in water and acetonitrile. The step gradient 4 was run with a flow rate of 0.25 mL/min. The injection volume was 2 µL. ESI MS was equipped with quadrupole ion trap. Capillary voltage was set at 4,500 V, nebulizer gas was set at 2 bars, and drying gas temperature was 220 ^{o}C with a flow rate of 7.0 L/min. MS

evaluation was performed in both positive and negative mode alternately and scanned at the mass range of m/z 70-900 amu. Chromelon and Hystar were used for controlling the system.

Quantitative methods for analysis of Triphala formulation

Preparation of standard solution

Gallic acid and rutin hydrate were prepared in the concentration of 1,000 µg/ml in methanol and dilute to 20, 40, 80, 160, 320 µg/ml and 5, 10, 20, 40, 80, 160 and 320 µg/ml (n=2), respectively. The solutions were filtered through 0.45µm syringe filter and injected to HPLC in triplicate. The standard curves were plotted between the concentrations and peak areas. The correlation coefficient was calculated.

Preparation of sample solution

Triphala formula and each plant were weighed accurately 50 mg and dissolved in 25 ml of 50%ethanol (n=2). The solution was filtered through 0.45µm syringe filter and injected to HPLC in triplicate. Peak areas of gallic acid and rutin were compared to the standard curves and calculated into their contents.

The HPLC analytical condition was performed using the step gradient 4. This method was validated and the data was summarized in the supplementary document.

Table 2. The gradient system used in chromatographic separation

Step gradient 3			Step gradient 4		
Time (min)	1% acetic acid in water (%v/v)	Acetonitrile (%v/v)	Time (min)	1% acetic acid in water (%v/v)	Acetonitrile (%v/v)
0	90	10	0	95	5
10	80	20	1	95	5
15	72	28	4	90	10
20	65	35	12	85	15
25	50	50	32	65	35
26	0	100	35	50	50
30	0	100	37	0	100
32	90	10	40	0	100
40	90	10	41	95	5
			45	95	5

RESULTS AND DISCUSSION

Reversed phase (RP) chromatography was developed and successfully eluted major compounds found in *T. chebula*, *T. bellirica*, *P. emblica* and Triphala formulations. The linear gradient with 1% acetic acid in water and either methanol or acetonitrile could not separate most compounds (chromatograms were not shown). However, acetonitrile showed better sensitivity and lower background noise including faster elution comparing with methanol. Thus it was used as a composition in the mobile phase of step gradient. Step gradient 3 resulted in much better separation (chromatograms were not shown) but still had low resolution at the beginning and in the middle of the run. Step gradient 4 demonstrated improve the resolution in the middle of the run. HPLC chromatograms of Triphala formula (T.F.1) are compared with the chromatograms of individual plants and modified formulations as shown in Figure 1, 2 and 3, respectively. HPLC condition was adapted from the chromatographic method which was used for characterization of the chemical constituents in four Terminalia species by Dhanani and *et al* (Dhanani, 2014). The order of elution was in similar pattern which was determined by photodiode array and ESI-MS. The maximal absorption wavelength of standard gallic acid is 272 nm and that of rutin is 256 and 354 nm. The absorbance values of these compounds in Triphala were in similar (~ 300 mAU);

therefore, the wavelength of 270 nm was used to monitor both compounds in this study. The standard quercetin was also checked but low content was found in all samples; therefore, it was not quantitative analysis in this study.

Mass spectra of the eluted compounds in Triphala were determined compared to the standard gallic acid and rutin. The molecular ions indicated the corresponding molecular weights in both positive and negative modes. The MS spectra of gallic acid, corilagin, chebulagic acid and chebulinic acid are shown in Figure 4-8. MS/MS fragmentation pattern was needed to evaluate rutin (M.W. 610.52) because the pH 2.65-2.67 of 1% acetic acid in water could cause some degradation of glycosides. Its base peak was m/z of 300.8 and a small molecular ion at m/z of 609 was seen in negative mode. Quercetin was seen as a tiny peak at the retention time of 32 minute. MS/MS fragmentation was also need to differentiate ellagic acid and quercetin due to the same molecular weights of 302.197 and 302.236, respectively. In this study, it was rarely seen ellagic acid peak in HPLC chromatograms of *T. chebula* and *T. bellirica* (Figure 2) comparing with the results by Dhanani and *et al*. This study showed that this optimized analytical method could be used for quality control of Triphala formulations which may contain varied ratios of herbal plants. The content of gallic acid could be used as the major marker because it was related to the activity and clearly found in all formulations and individual plants.

The gallic acid and rutin contents in Triphala and modified formulations were determined using HPLC analysis. The standard curves of gallic acid (y=61774x + 71944) and rutin (y=34293x + 9555) showed linear correlations with correlation coefficients (R^2) of 0.9993 and 0.9996, respectively. Their contents were reported in %w/w as shown in Table 3. There were not significantly different gallic acid contents among these formulations. *P. emblica* and M.T.F.3 and M.T.F.4 showed the highest gallic acid contents while *T. chebula* and M.T.F.3 showed the highest rutin contents. The gallic acid content of the individual herb was not less than 1%w/w on dry basis by HPLC method according to the Thai herbal pharmacopeia criteria. The gallic acid contents (1.32-4.02 %w/w) were slightly higher than the result (1.07-2.44 %w/w) which was analyzed by TLC densitometry.

Table 3. Gallic acid and rutin contents in Triphala formulations and each plant

Formula/herbal plant	Gallic acid content (%w/w)	Rutin content (%w/w)
T.F. 1	2.66 ± 0.01	2.05 ± 0.00
MT.F. 2	2.67 ± 0.02	2.39 ± 0.05
MT.F. 3	2.87 ± 0.03	2.94 ± 0.01
MT.F. 4	2.87 ± 0.04	1.51 ± 0.10
MT.F. 5	2.70 ± 0.00	0.65 ± 0.00
T. chebula	2.37 ± 0.00	2.61 ± 0.04
T. bellirica	1.32 ± 0.03	0.61 ± 0.70
P. emblica	4.02 ± 0.02	1.26 ± 0.03

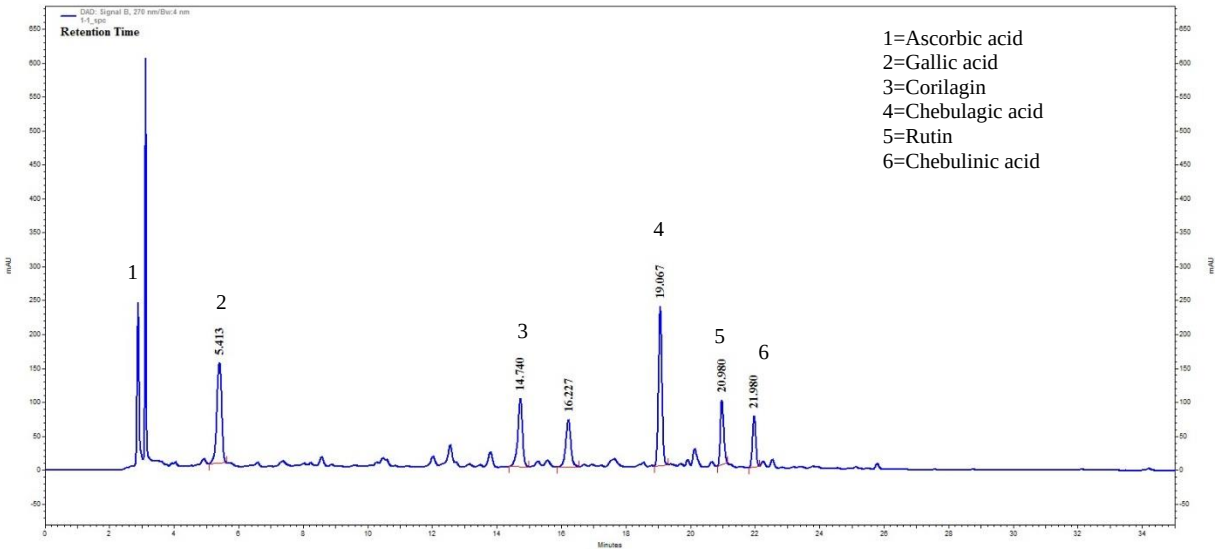

Figure 1. HPLC chromatogram of Triphala (T.F.1) at the wavelength of 270 nm

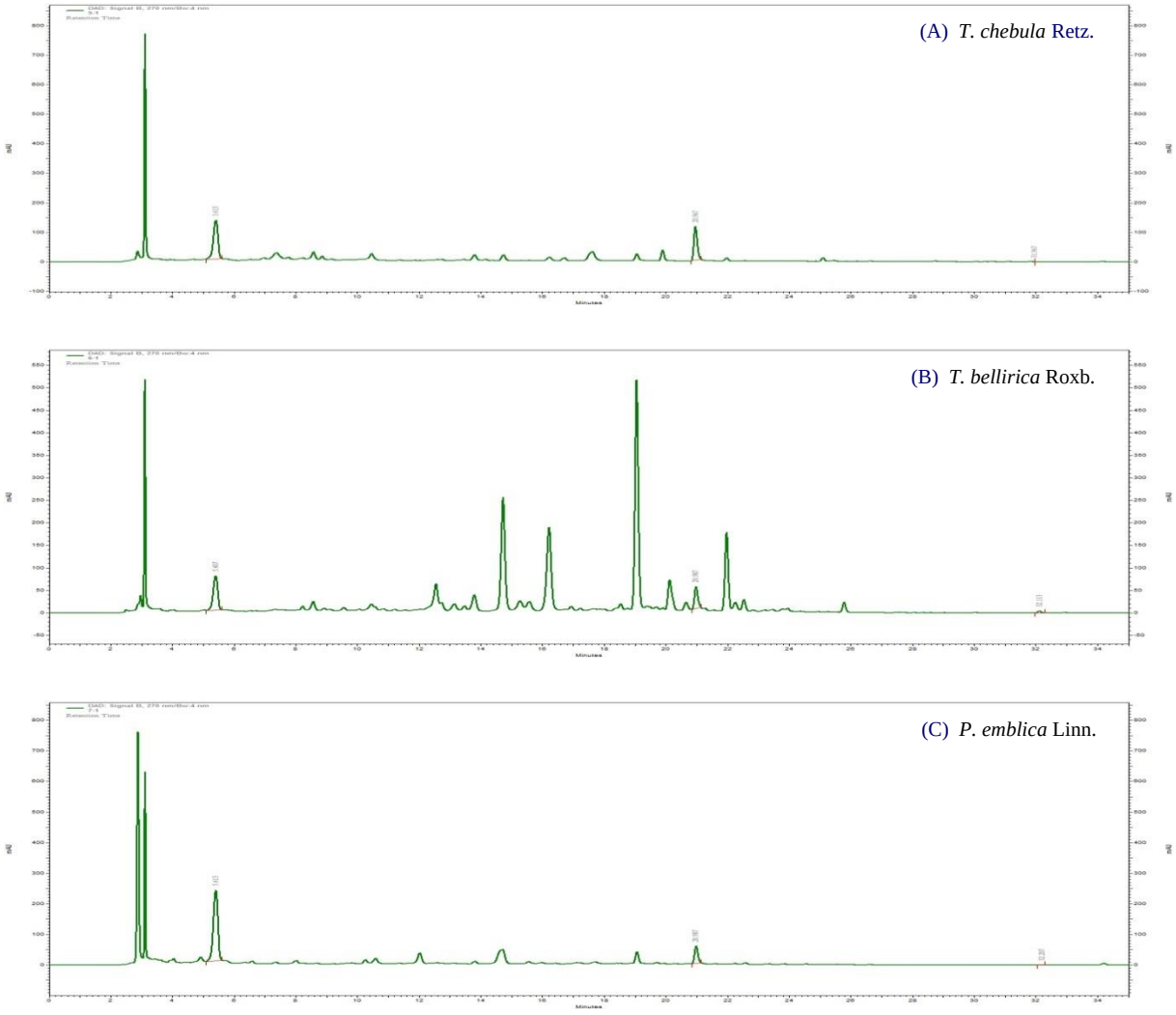

Figure 2. HPLC chromatograms of *T. chebula* , *T. bellirica* , *P. emblica* at the wavelength of 270 nm

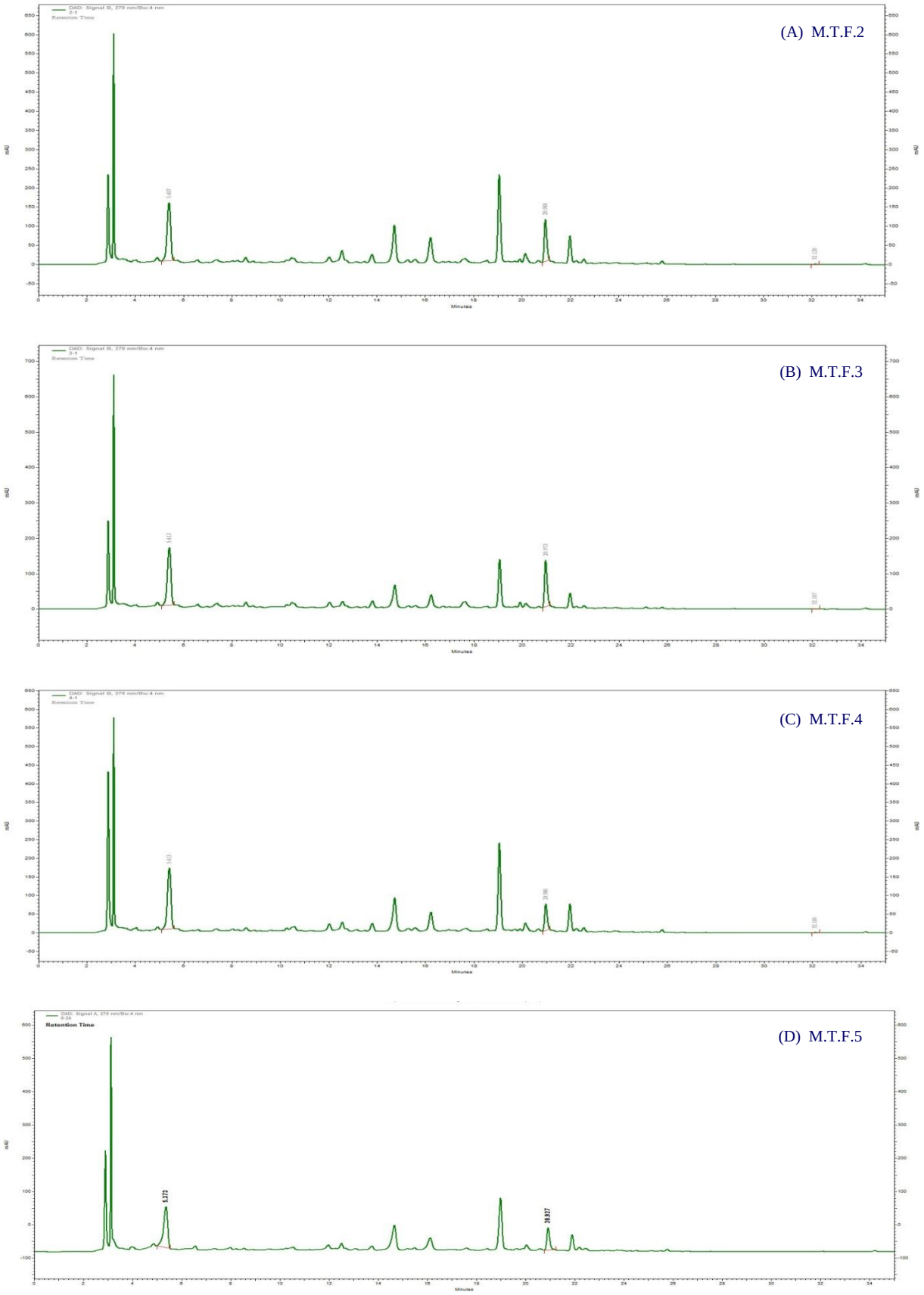

Figure 3. HPLC chromatograms of Modified Triphala Formulations at the wavelength of 270 nm

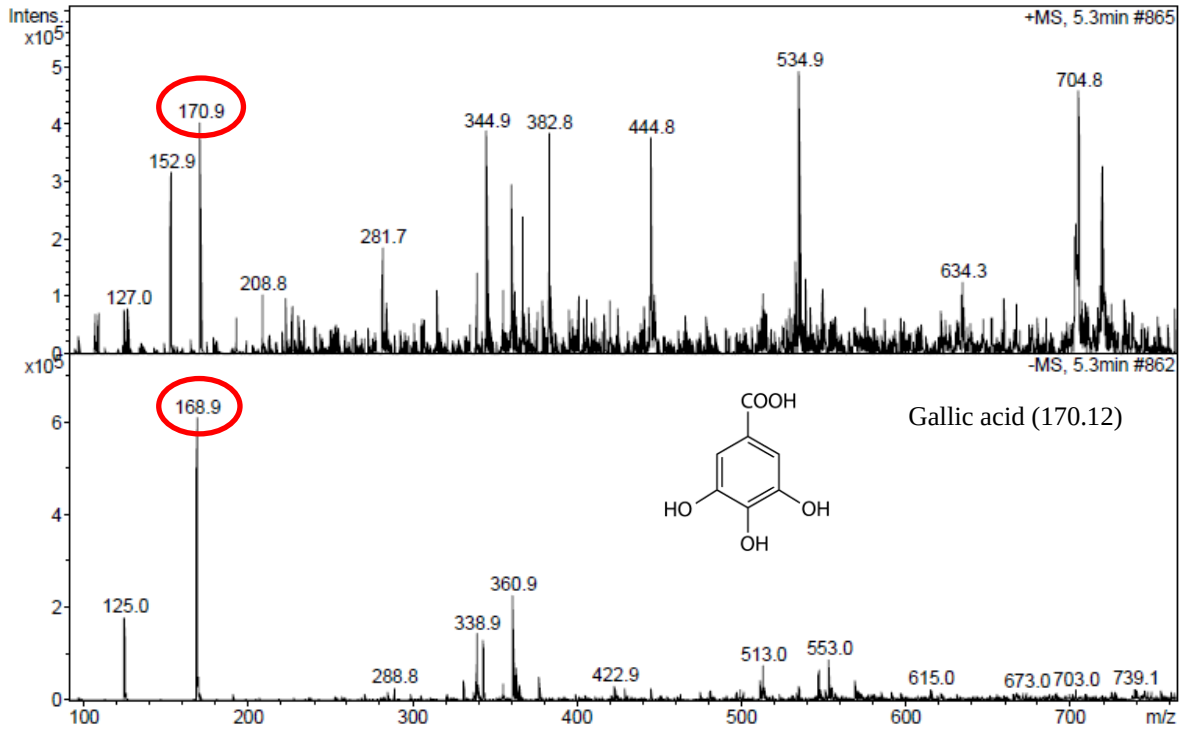

Figure 4. MS spectra of gallic acid found in T.F.1

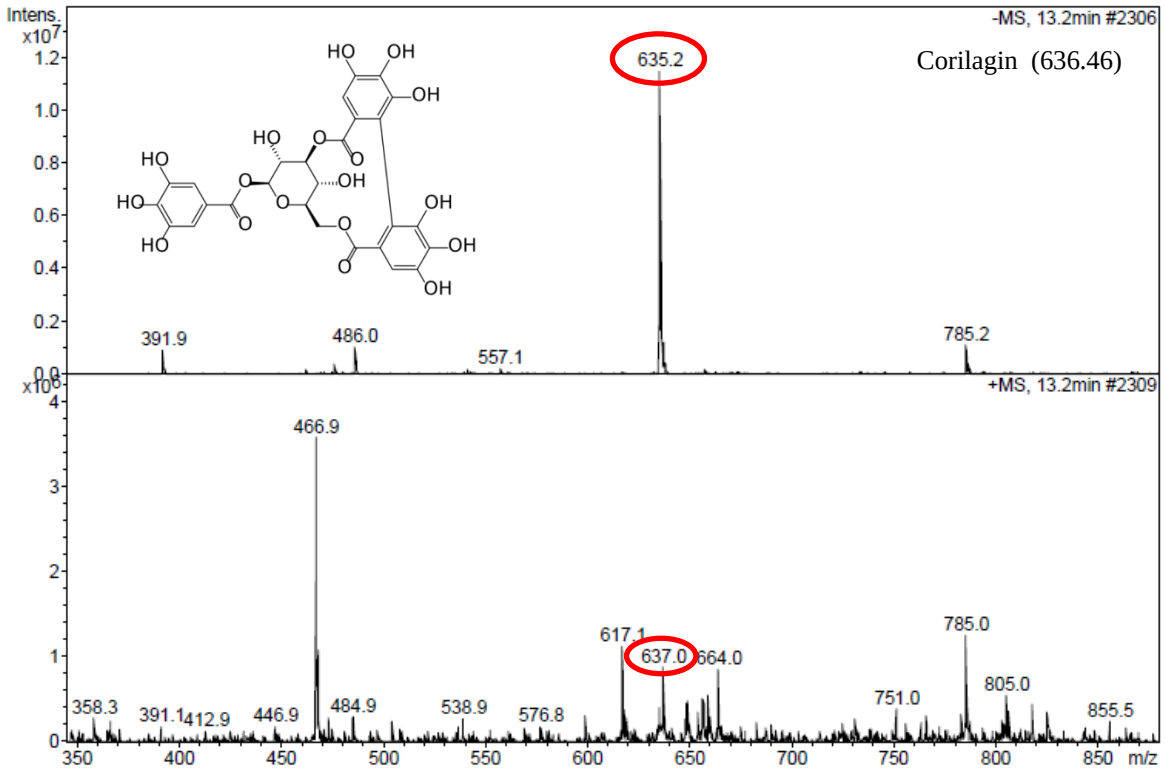

Figure 5. MS spectra of corilagin found in T.F.1

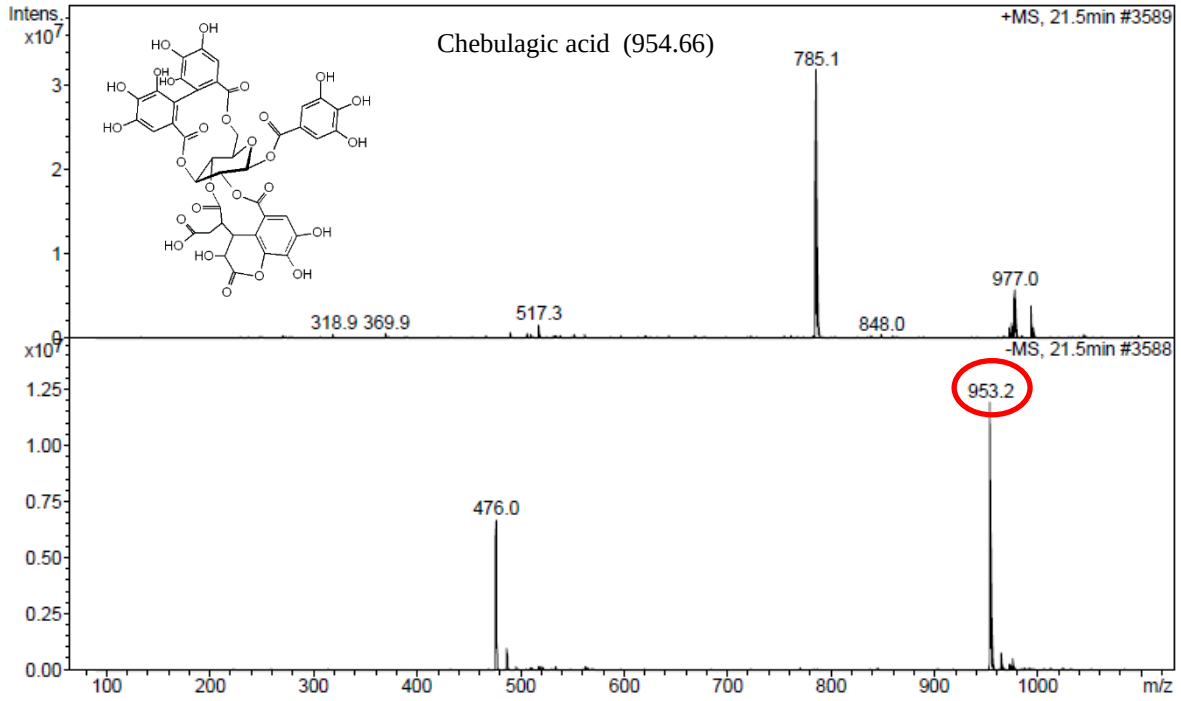

Figure 6. MS spectra of chebulagic acid found in T.F.1

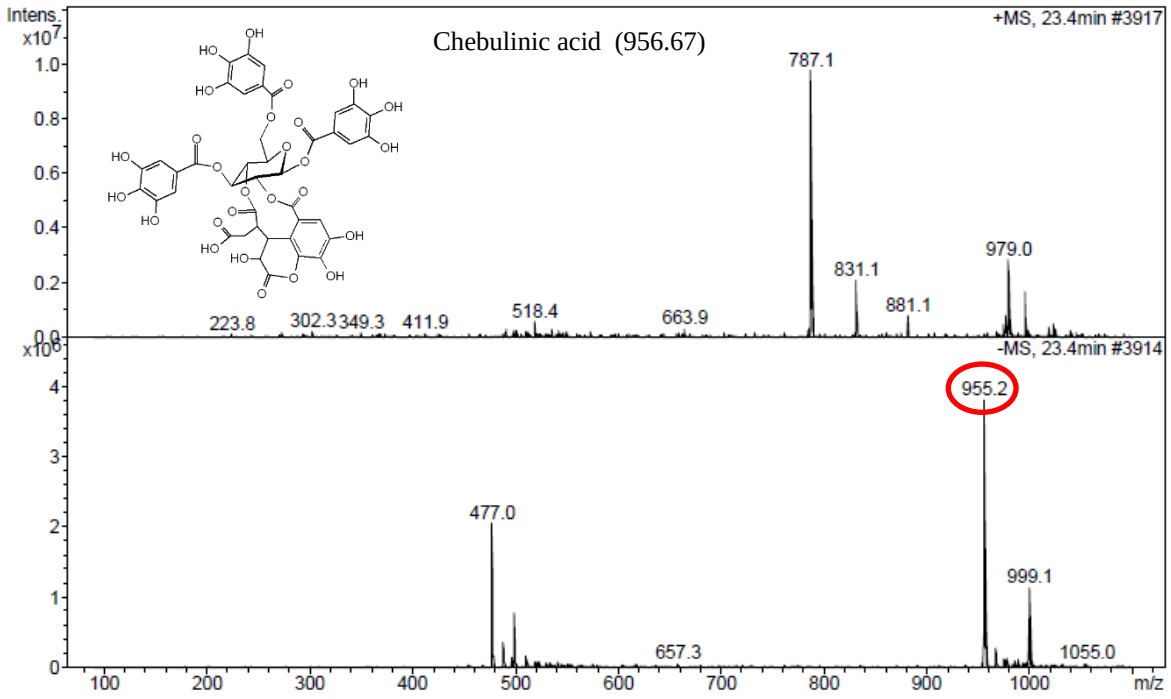

Figure 7. MS spectra of chebulinic acid found in T.F.1

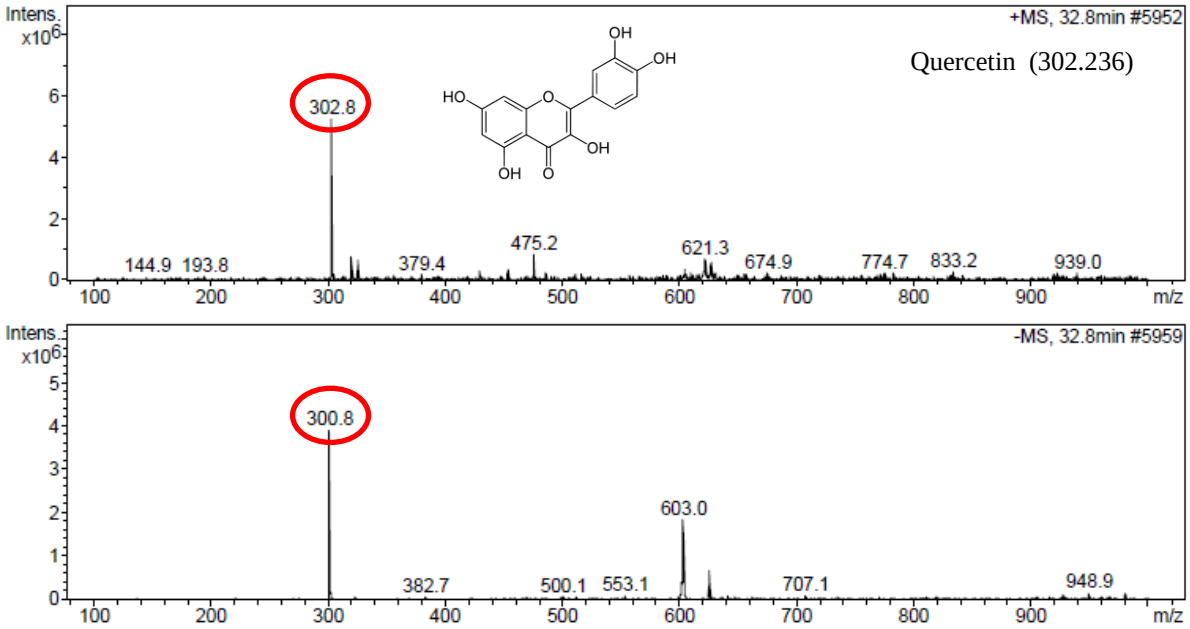

Figure 8. MS spectra of quercetin found in T.F.1

CONCLUSION

RP HPLC was successfully determined main constituents in Triphala formulations using simple mobile phase composition and step gradient run. ESI MS identified these compounds and the order of elution was ascorbic acid, gallic acid, corilagin, chebulagic acid, rutin, chebulinic acid and quercetin. The gallic acid and rutin contents were 1.32-4.02 and 0.61-2.94 % w/w, respectively. ESI MS in positive and neagative modes were useful to identify the molecular weights of chemical compounds found in Triphala. However, MS/MS fragmentation will need to be undertaken to differentiate the compounds with the same molecular weight.

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