Chemical Profiling of an Antipyretic Drug, Thai Herbal Harak Formula, by Liquid Chromatography Coupled with Quadrupole Time-of-Flight Mass Spectrometry

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ABSTRACT

Objective: To isolate and identify chemical compounds in Harak, a Thai herbal formula widely used as an antipyretic drug in Thailand.

Methods: Methanol extraction of the Harak formula and its five herbal components were separated by Ultra high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry. This method has been developed for "untargeted" profiling of this formula and its components. An online database was used to identify chemical compounds by comparing its empirical molecular formula, isotope pattern, and fragmentation pattern.

Results: Nineteen chemical constituents were found from positive and negative electrospray ionization (ESI) modes. These compounds included flavonoids, hesperitine, and iso-corydine, which are known to possess antioxidant and anticancer activity. Moreover, data from the principle component analysis (PCA) score plot of positive and negative ESI modes showed that the chemical constituents of Thai herbal Harak formula were similar to those found in *Ficus racemosa* Linn. and *Capparis micracantha* DC.

Conclusion: Under this optimization method, nineteen chemical constituents including phenolic and flavonoids were characterized in both positive and negative ESI mode.

Keywords: Chemical profiling; Thai herbal Harak formula; Thai traditional medicine; time-of-flight mass spectrometry; ultra high performance liquid chromatography (Siriraj Med J 2018;70: 159-168)

INTRODUCTION

A Thai herbal Harak formula (HRF) or Ha-Rak recipe has been widely used in Thailand as an antipyretic drug. The formula has been included in the essential drug list of herbal medicinal products since 2006 by National Drug Committee of Thailand.[1] HRF is prepared by the combination of five dried roots powder in the equal parts by weight; *Ficus racemosa* Linn. (FR), *Capparis micracantha* DC. (CP), *Harrisonia perforata* (Blanco) Merr. (HP), *Tiliacora triandra* (Colebr.) Diels. (TT) and *Clerodendrum petasites* (Lour.) S. Moore (CP). Pharmacological activities of HRF have been previously studied such as antipyretic[2], antioxidant[3] and anti-inflammatory.[4] Methanol and aqueous extracts of Ben-Cha-Lo-Ka-Wi-Chian remedy; another name for HRF, had antipyretic and antinociceptive properties in animal models[2]. Ethanol extracts of HRF and its herbal components suppressed UVA-induced matrix metalloproteinase-1 (MMP-1) activity in keratinocyte HaCaT cells. Moreover, they regulated the endogenous antioxidants including

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glutathione (GSH), catalase, and glutathione peroxidase (GPx).[3] Previous study also showed non-direct mutagenic activity of this formula.[4] Many scientific studies[5-17] have suggested that the main chemical constituents of each component of HRF, excluding CM, were phenolic acid and flavonoids which are known as pharmacological substances of antioxidant, anti-inflammatory and anticancer actions. However, scientific data that support the chemical constituents in a polyherbal formula HRF was rare. Until now, there was only one work that studied chemical components from HRF remedy. Singharachai C, et al. 2011[4] had found twelve components (Cappine B, Yanangine, Tiliacosine, Tiliacorinine, Tiliasine, Nevedencin 7-rutinoside, Hispidulin 7-methyglucoside, Hispidulin, Heteropeucenin 7-Me-ether, Perforatin G, Racemosa and Isowingtione) from HRF remedy using 3D-HPLC followed with UV spectrum patterns data comparison.

In the past decades, many reports had been developed the best way for isolation and identification of chemical constituents from herbal medicines (HMs). However, HMs comprise hundreds of different constituents that belong to numerous compound classes.[18] Moreover, the challenging task of HMs is some HMs contain two or more than two herbal components. These HMs are more complex, therefore, analysis of HMs are more difficult. The analytical selectivity and sensitivity of liquid chromatography coupled with mass spectroscopy (LC-MS) together make the most selective technique for rapid screening and characterization of known and unknown constituents from the HMs. Nowadays, Liquid Chromatography Quadrupole Time-of-flight Mass Spectrometry (LC-Q-TOF) is one of the powerful tools to isolate and identify chemical constituents in HMs. LC-Q-TOF provides accurate mass measurements (possibility to yield mass accuracy <5 ppm with an adequate calibration range)[19, 20] and high resolutions. The accurate mass measurement from LC-Q-TOF gives elemental composition of both parent and fragment ions.[19, 21-23] Thus, it is appropriate for identification of unknown constituents. There are many applications of LC-Q-TOF for HMs analysis such as qualitative, quantitative of HMs or study of metabolites. HMs include known and unknown chemical compounds. An interesting function of LC-Q-TOF for HMs qualitative analysis is identification of complex chemical constituents which involves the confirmation of targeted compounds and elucidation of non-targeted compounds. To screen non-targeted compounds from HMs using LC-Q-TOF, the exact elemental compositions of parent ions are generated from several steps which are applied from Chen XF, et al. 2011.[20] LC-Q-TOF software, MarkerLynx™ XS (Waters Corp., MA, USA), generated the possible molecular composition according to accurate mass measurements after low fragmentation voltages. Then, the most probable molecular composition was selected and searched against an on-line chemical library. Finally, the library match was recorded for each chemical formula. Because of the complexity of HMs, only parent ions identification may not be enough for confirmation. Therefore, fragment ions of parent ion are generated using high fragmentation voltages and the software of Q-TOF, MassFragment™ software (Waters Corp., MA, USA). This provides valuable structural information by producing various characteristic fragment ions together with their elemental compositions.

The present study, developed the first chemical profiling of a Thai herbal Harak formula and its five herbal components. LC-Q-TOF was used as a tool for isolation and identification of chemical profiling. Due to the complexity and lacking of chemical constituents data in HRF, chemical compositions of HRF were generated by screening method. Moreover, we focussed on the most possible chemical targets, therefore mass spectrometry detection was performed in both positive and negative electrospray ionization mode (ESI). The most complete datasets were identified by matching and comparison of empirical molecular formula with online database. This work provided the rapid and reliable method for isolation and identification of the chemical profiling in HRF and the other HMs.

MATERIALS AND METHODS

Chemicals solvents and herbal materials

LC-MS grade acetonitrile, methanol, formic acid and analytical grade ethanol were purchased from Scharlau (Spain). Purified water was prepared by a Milli-Q water system (Millipore, France).

HRF and its five herbal components (FR, CM, HP, TT and CP) were prepared by the good manufacturing practice (GMP) certified Manufacturing Unit of Herbal Medicines and Products, Center of Applied Thai Traditional Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. All of them were authenticated by experienced Thai traditional practitioners. Then, the powders were stored and kept at room temperature in air-dried condition. The five herbal components of HRF were shown in Fig 1.

Preparation of HRF and its five herbal components

For identification of chemical constituents, the HRF, FR, CM, HP, TT and CP powders were accurately weighed (100 mg) and ultrasonically extracted with methanol 1 mL for 60 min. Each extracted solution was centrifuged

Fig 1. Five dried roots of HRF's herbal components: (A) *Ficus racemosa* Linn., (B) *Capparis micracantha* DC., (C) *Harrisonia perforata* (Blanco) Merr., (D) *Tiliacora triandra* (Colebr.) Diels. and (E) *Clerodendrum petasites* (Lour.) S. Moore.

(Eppendorf, Germany) at 12,000 rpm for 10 min at 4°C and the supernatant was filtered through 0.2 µm polyvinylidenedifluoride (PVDF) syringe filter (Vertical Chromatography, Thailand). The final concentrations of all sample solutions with 10 mg/mL and 4 µL were injected into UHPLC-Q-TOF.

Chromatographic conditions

Chromatographic analysis was performed with a Waters ACQUITY UPLC® system (Waters Corp., MA, USA) equipped with a binary solvent delivery system, an online degasser, an auto sampler and a thermostatically controlled column system. The separation was performed on a Waters ACQUITY HSS PFP (100 mm × 2.1 mm, 1.8 µm, Waters, Ireland) column at 30°C. The auto sampler was maintained at 10°C. The mobile phase consisted of (A) 0.1% formic acid in purified water and (B) 0.1% formic acid in acetonitrile using an optimization linear gradient elution of 2-99%B over 0-9.0 min, kept at 99%B for 1 min, 99-2%B over 10.0-12.0 and kept at 2%B for 3 min. The sample volume injected was 4 µL and the flow rate was 400 µL/min.

Mass spectrometry detection was performed on Waters® Xevo™ Q-TOF Mass spectrometer (Waters Corp., MA, USA) equipped with ESI source. The analysis carried out by positive ion and negative ion mode of MS^E analysis mode, involving low collision energy of 4 V for the precursor analysis and for the fragment ions the analysis was performed using high collision energy of 20-40 V. Mass range was set as m/z 100-1,000. The source parameters were electrospray capillary voltage 3 kV, source temperature at 120°C and desolvation temperature at 600°C. The cone voltage was set at 20 V. Nitrogen and argon were used as cone and collision gases, respectively. The cone and desolvation gas flow were set at 50 L/hr and 900 L/hr respectively.

To ensure the accuracy and reproducibility of all analysis, a reference standard lock spray interference, leucine encephalin (Waters Corp., MA, USA) was used. The lock mass of leucine encephalin for positive mode and negative mode were 556.2771 and 554.2615 respectively, at a concentration 200 pg/mL, with the infusion flow rate of 20 µL/min and scan injection every 30 sec.

Data processing and statistical analysis

The MassLynx™ V4.1 software (Waters Corp., Milford, USA) were used to collecting all data. The EZinfo software (Umetrics UK Ltd, UK) was used for multivariate statistical analysis; PCA (principal component analysis). The possible markers were extracted from S-plot of the OPLS-DA (an orthogonal partial least-squares-discriminant analysis). To avoid the chemical noise, pareto scaling was used in all the models. The MarkerLynx™ XS (Waters Corp., Milford, USA) and MassFragment™ software (Waters Corp., Milford, USA) were used for identifying chemical markers which were confirmed by mass spectrum and chromatographic retention times following databases, such as ChemSpider, Natural Remedies, KEGG and Pubmed.

RESULTS

Chromatographic conditions and Q-TOF method development

Preliminarily, these chromatographic conditions were tested on four columns. The ACQUITY UPLC® HSS PFP (100 mm × 2.1 mm, 1.8 µm, Waters, Ireland) had a good separation and gave a large number of compounds thus it was chosen for this study. The other columns were Waters ACQUITY BEH Shield RP18 (100 mm × 2.1 mm, 1.8 µm), Waters ACQUITY CSH C18 (100 mm × 2.1 mm, 1.8 µm) and Waters ACQUITY HSS T3 (50 mm × 2.1 mm, 1.8 µm). A solvent system consisted of 0.1% formic acid in purified water and acetonitrile provided the superior separation and baseline stability. Gradient elution was used for the best separation in 10 min at flow rate 400 µL/min.

Mass spectrometry detection was performed on a Waters® Xevo™ Q-TOF mass spectrometer equipped with ESI source. Untargeted analysis was applied for identify abundant chemical constituents in HRF and its components. Since there was no target analysis, samples should be separated as much as possible thus the separations were in both positive and negative ionization mode. Analyses of the other parameters were conducted to get more peak and fragmentation information for further identification of the chemical constituents.

Chemical profiling of HRF and its herbal components

This work developed the first chemical profiling of HRF and its five herbal components; FR, CM, HP, TT and CP, by LC-Q-TOF. HRF and all herbal components were separated by UHPLC-Q-TOF, which in this case the ionizations were done in both positive and negative mode. Chemical profiling of HRF (a.), FR (b.), CM (c.), HP (d.), TT (e.) and CP (f.) in positive and negative ionization mode were exhibited in Fig 2A and 2B respectively. All of the chemical profiling showed in base peak intensity (BPI) chromatograms. Both positive and negative ESI mode demonstrated the complexity of chemical constituents in HRF and all herbal components. The UHPLC-Q-TOF system over the m/z 100-1,000 within 12 min could detect many peaks.

Peak assignment from HRF and its herbal components

Currently, chemical constituent data of HRF were less and remain unclear. Most of HRF's herbal components were found to be phenolic acid and flavonoids. For this study, we would like to find some chemical constituents from HRF and its components. Therefore, "Untargeted" screening method was applied to generate chemical constituents from HRF and each herbal component. In order to obtain the chemical data, principal component analysis (PCA) was utilized to depict the different chemical constituents between the HRF and its five herbal components. PCA Score plot of HRF and its five herbal components in positive and negative ESI mode were shown in Fig 3A and 3B respectively. As shown in the PCA Score plot of positive and negative ESI modes, each sample was represented as a point. The HRF, FR, CM, HP, TT and CP were clearly separated as the principal components in both positive and negative ESI mode. The red circles in PCA exhibited that chemical constituents of HRF were similar to those found in FR and CM by the principal component 3 (PC3) and 4 (PC4). In addition, the key chemical constituents were generated

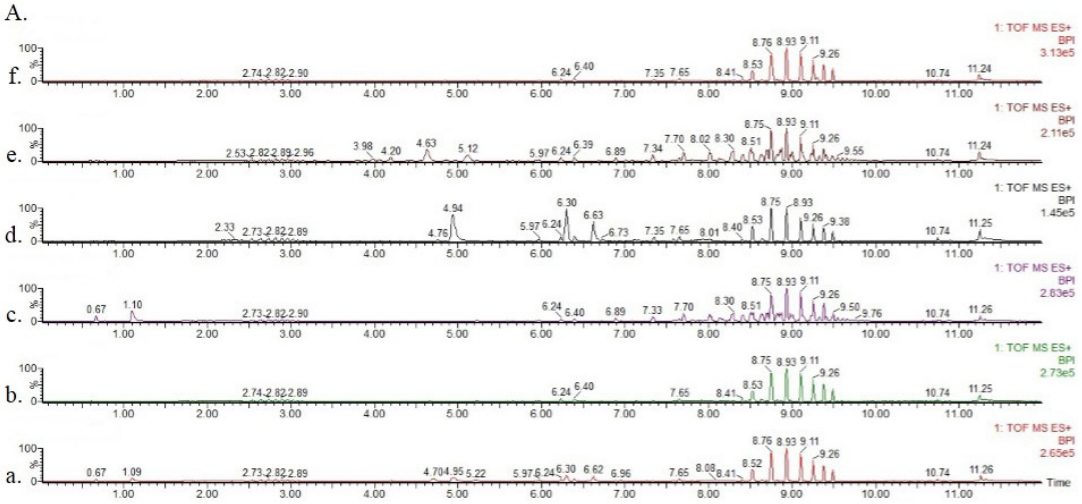

Fig 2A.

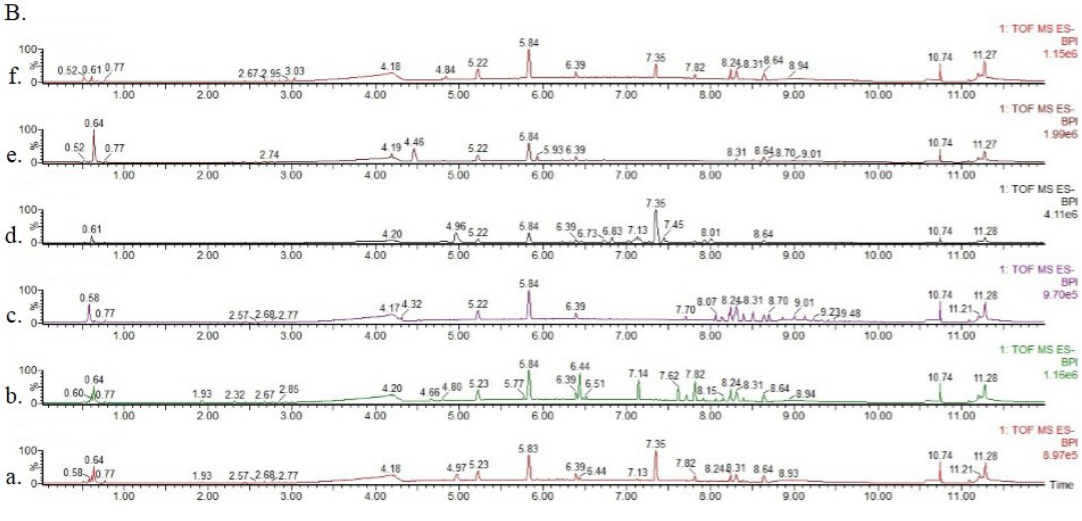

Fig 2B.

Fig. 2 Base-peak ion (BPI) chromatograms of HRF and its five herbal components from LC-QTOF analysis with (A) positive ESI mode and (B) negative ESI mode: a) HRF, b) *Ficus racemosa* Linn., c) *Capparis micracantha* DC., d) *Harrisonia perforata* (Blanco) Merr., e) *Tiliacora triandra* (Colebr.) Diels. and f) *Clerodendrum petasites* (Lour.) S. Moore.

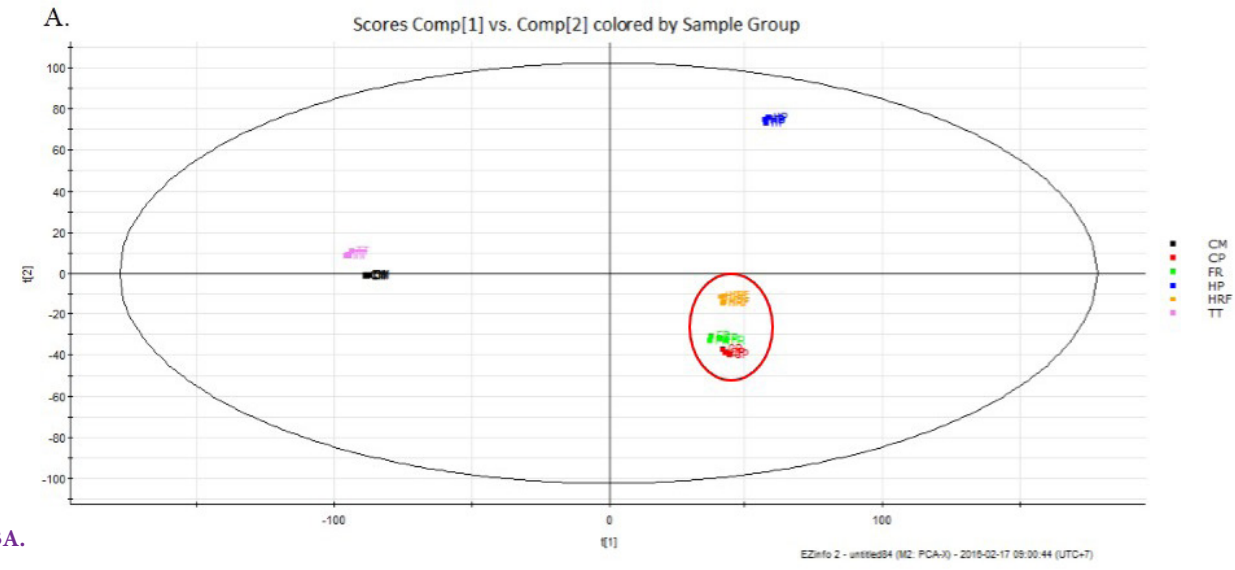

Fig 3A.

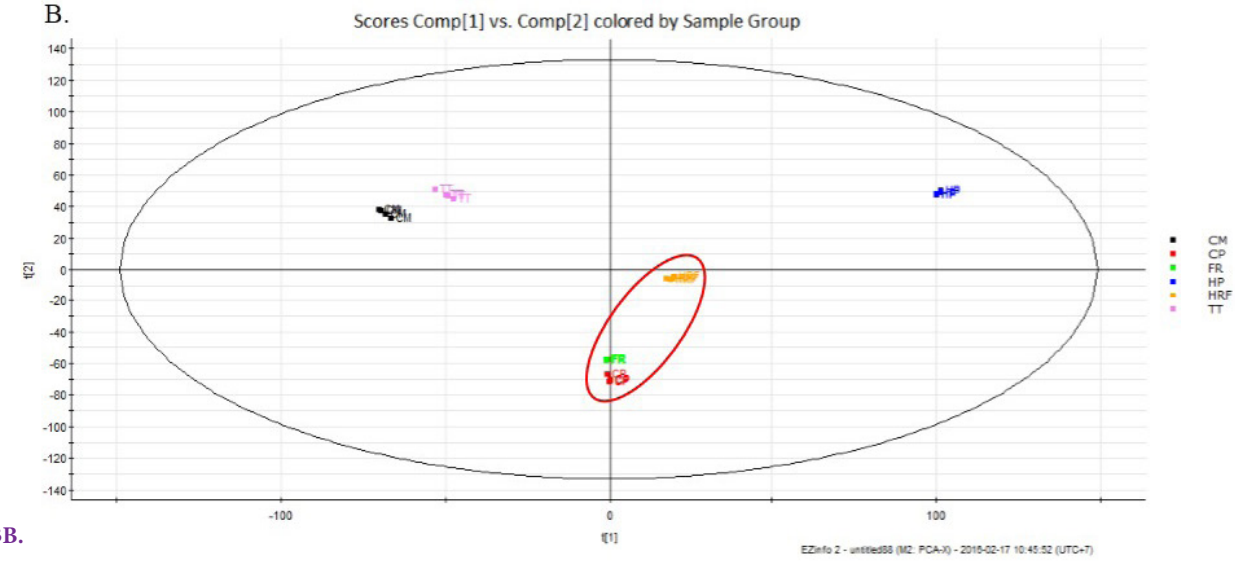

Fig 3B.

Fig. 3 PCA Score plot of HRF and its five components in (A) positive ESI mode and (B) negative ESI mode; Thai herbal Harak formula (HRF), *Ficus racemosa* Linn. (FR), *Clerodendrum petasites* (Lour.) S. Moore (CM), *Harrisonia perforata* (Blanco) Merr. (HP), *Tiliacora triandra* (Colebr.) Diels. (TT) and *Capparis micracantha* DC. (CP).

by OPLS-DA S-plot (Fig 4 and Fig 5 for positive and negative ESI mode respectively). Identification of the differences between sample groups was elucidated by MarkerLynx™ XS data processing which then generated the key chemical targets. All chromatograms in each positive and negative ESI mode were aligned. Possible key chemical markers were created by correlation and coefficient value of OPLS-DA S-plot. Each sample was compared together with the rest samples. In Fig 4 and Fig 5, marker ions had correlation value nearly -1 and coefficient value far from the center of OPLS-DA S-plot, suggesting that was the more important compound. These candidate markers were tentatively identified by isotope pattern matching and comparison of empirical molecular formula with online database (Chembank, ChemDB, KEGG, Natural Remedies, Nature Chemical Biology, Nature Chemistry and Pubmed). Moreover, for structural elucidation conformation, fragmentation pattern matching was done by MassFragment™ software.

Nineteen chemical constituents including phenolic and flavonoids which are known as pharmacological substances of anti-fungal and anticancer activities,[24,25] were found from positive and negative ESI mode. The data showed only compounds which matched with online database. Table 1 showed eight candidate compounds found in both HRF and its herbal components in positive ESI mode. In negative ESI mode, twelve candidate compounds were identified from both HRF and its herbal components (Table 2). Surprisingly, 5,7-Dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-4H-chromen-4-one or hesperitine was found in both positive and negative ESI modes and expressed in all HRF's five components.

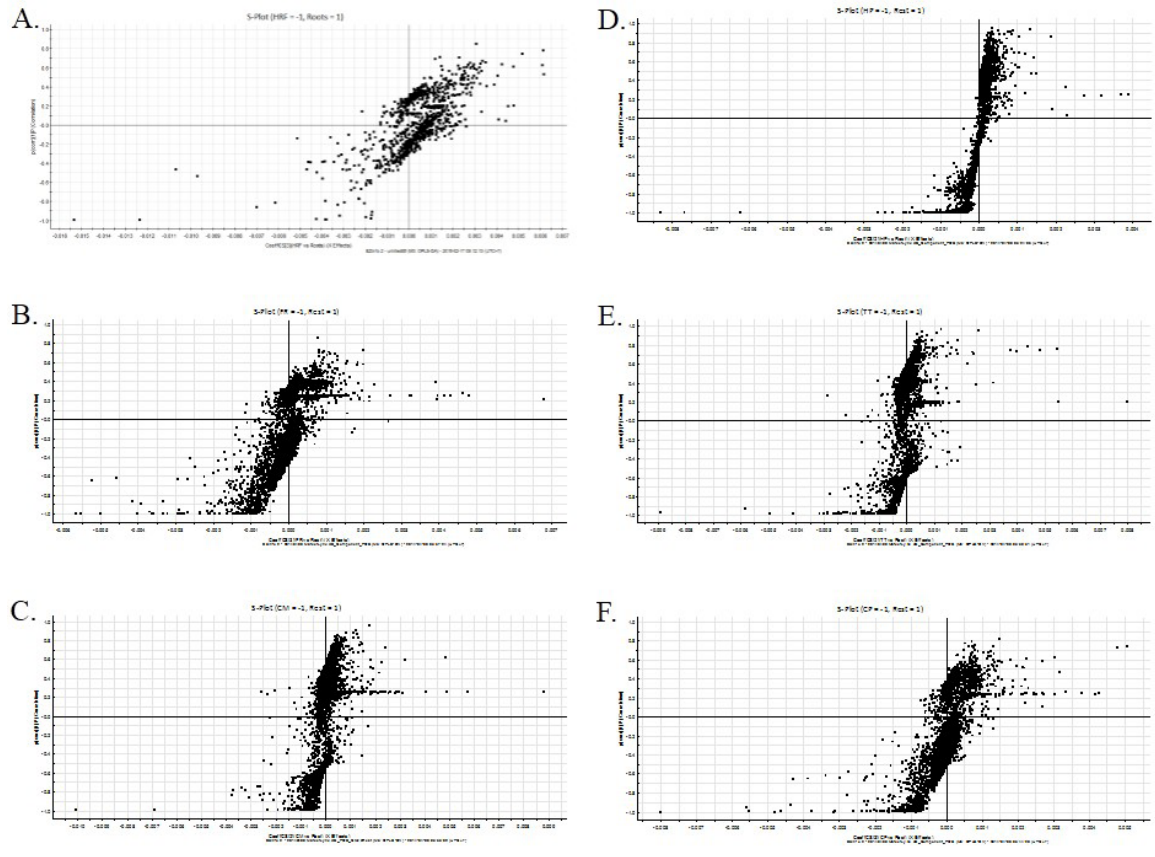

Fig 4. OPLS-DA S-plot of HRF and its five herbal components in positive ESI; HRF (A), *Ficus racemosa* Linn. (B), *Clerodendrum petasites* (Lour.) S. Moore (C), *Harrisonia perforata* (Blanco) Merr. (D), *Tiliacora triandra* (Colebr.) Diels. (E) and *Capparis micracantha* DC. (F).

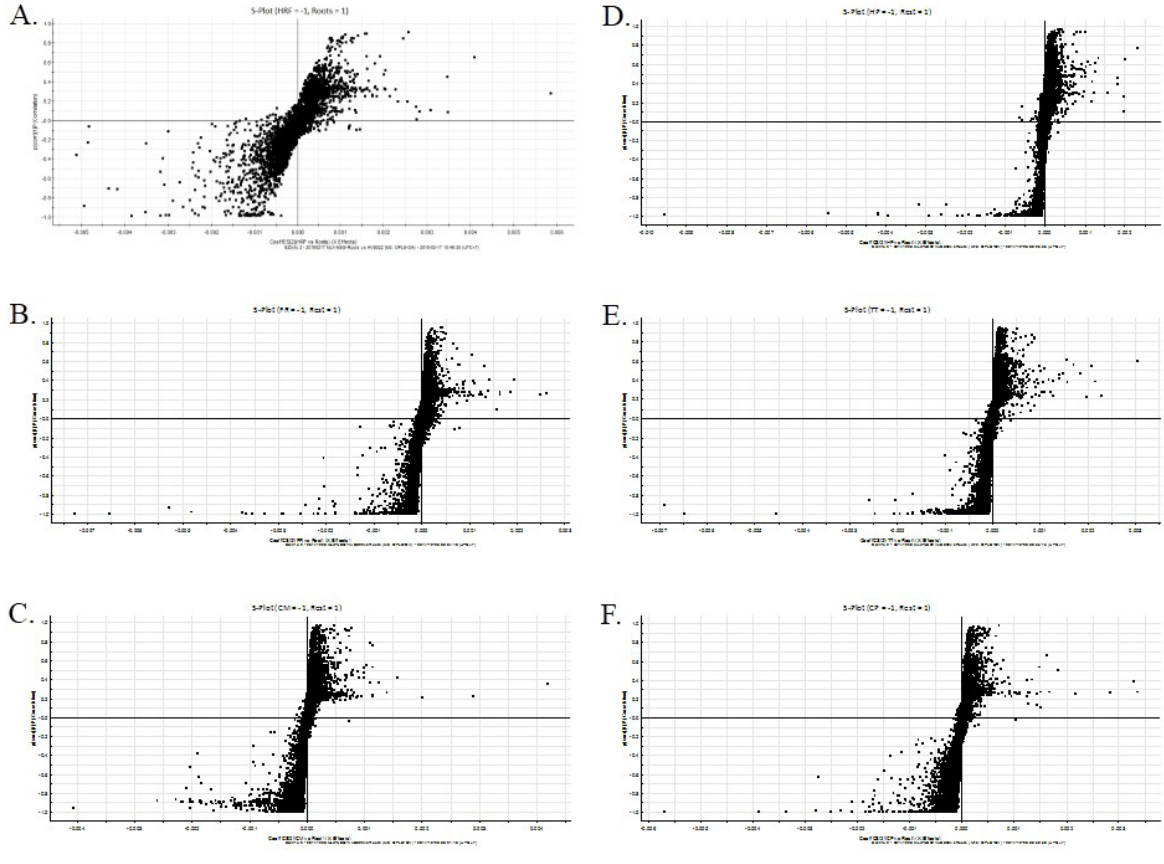

Fig 5. OPLS-DA S-plot of HRF and its five herbal components in negative ESI; HRF (A), *Ficus racemosa* Linn. (B), *Clerodendrum petasites* (Lour.) S. Moore (C), *Harrisonia perforata* (Blanco) Merr. (D), *Tiliacora triandra* (Colebr.) Diels. (E) and *Capparis micracantha* DC. (F).

TABLE 1. Tentatively identified of compounds from HRF and its five components using LC-QTOF in positive ESI mode.

RT(min)	Parent ions (m/z)	Actual mass	Tentative identification	Elemental composition	HRF	FR	CM	HP	TT	CP
0.6684	160.0972 $[M+H]^+$	159.0894	Methyl 2-[(isopropylideneamino)oxy] propanoate	$C_7H_{13}NO_3$	/		/			
1.1061	144.1023 $[M+H]^+$	143.0945	1-(Hydroxymethyl)-2-azepanone	$C_7H_{13}NO_2$	/		/			
2.5310	388.2544 $[M+H]^+$	387.2465	N-{[(2-Methyl-2-propanyl)oxy]carbonylleucyl-N~5~-(diaminomethylene) ornithine	$C_{17}H_{33}N_5O_5$	/	/	/	/	/	/
4.2009	314.1396 $[M+H]^+$	313.1318	Ethyl 11-oxo-2,3,6,7-tetrahydro-1H,5H,11H-pyrano[2,3-f]pyrido[3,2,1-ij] quinoline-10-carboxylate or Coumarin 314	$C_{18}H_{19}NO_4$	/				/	
4.8512	301.0714 $[M+H]^+$	300.2629	5,7-Dihydroxy-2-(4-hydroxyphenyl)-6-methoxy-4H-chromen-4-one or Hispidulin	$C_{16}H_{12}O_6$	/					/
4.7225	342.1710 $[M+H]^+$	341.1631	(6aS)-1,2,10-Trimethoxy-6-methyl-5,6,6a,7-tetrahydro-4H-dibenzo[de,g]quinolin-11-ol or iso-Corydine	$C_{20}H_{23}NO_4$	/					
4.9435	303.0876 $[M+H]^+$	302.0798	5,7-Dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-4H-chromen-4-one or Hesperitine	$C_{16}H_{14}O_6$	/			/		
6.2992	273.1133 $[M+H]^+$	272.1055	4,4'-(1,2-Hydrazinediylidene)bis (4,5,6,7-tetrahydro-2,1,3-benzoxadiazole)	$C_{12}H_{12}N_6O_2$	/			/		

Abbraviations: FR: *Ficus racemosa* Linn., CM: *Clerodendrum petasites* (Lour.) S. Moore, HP: *Harrisonia perforata* (Blanco) Merr., TT: *Tiliacora triandra* (Colebr.) Diels. and CP: *Capparis micracantha* DC.

TABLE 2. Tentatively identified of compounds from HRF and its five components using LC-QTOF in negative ESI mode.

RT(min)	Parent ions (m/z)	Actual mass	Tentative identification	Elemental composition	HRF	FR	CM	HP	TT	CP
0.5892	332.0107 $[M-H]^-$	333.0185	1-S-[N-(Sulfooxy) ethanimidoyl]-1-thiohexopyranose	$C_8H_{15}NO_9S_2$	/		/			
0.6462	191.0556	192.0634	1-Oxaspiro[2.5]octane-4,5,6,7,8-pentol	$C_7H_{12}O_6$	/			/	/	
4.1972	312.1235 $[M-H]^-$	313.1313	1,3,7-Trimethyl-8-[2-(2-pyridinylmethylene)hydrazino] -3,7-dihydro-1H-purine-2,6-dione	$C_{14}H_{15}N_7O_2$	/				/	
4.6585	269.0449 $[M-H]^-$	270.0527	1-[(4-Methylphenyl)sulfonyl]-2-(methylsulfanyl)-4,5-dihydro-1H-imidazole	$C_{11}H_{14}N_2O_2S_2$	/	/				
4.9827	301.0704 $[M-H]^-$	302.0782	5,7-Dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-4H-chromen-4-one or Hesperitine	$C_{16}H_{14}O_6$	/	/	/	/	/	/
5.7725	337.1072 $[M-H]^-$	338.1150	3-(1-Methyl-1H-pyrrol-2-yl)-N'-(3-nitrobenzylidene)-1H-pyrazole-5-carbohydrazide	$C_{16}H_{14}N_6O_3$	/	/				
5.8380	277.1808 $[M-H]^-$	278.1886	[2,6-Bis(dimethylamino)-4-pyrimidinyl](1-piperazinyl) methanone	$C_{13}H_{22}N_6O$	/	/				/
5.9324	295.2273 $[M-H]^-$	296.2352	1-Ethyl-4-{1-[1-(2-methoxyethyl)-1H-tetrazol-5-yl]-2-methylpropyl} piperazine	$C_{14}H_{28}N_6O$	/	/			/	/
6.5171	403.1543 $[M-H]^-$	404.1621	2-(2,3-Dimethoxyphenyl) cycloheptyl 4-methylbenzenesulfonate	$C_{22}H_{28}O_5S$	/	/				
6.5236	349.1077 $[M-H]^-$	350.1155	2-Oxo-3-(3-oxo-1-phenylbutyl)-2H-chromen-4-yl acetate	$C_{21}H_{18}O_5$	/	/				
6.4405	405.1699 $[M-H]^-$	406.1777	2-{[2-(1H-Indol-3-yl)ethyl] amino}-4',6'-dimethyl-6-oxo-5,6-dihydro-4H-1,2'-bipyrimidine-4-carboxylic acid	$C_{21}H_{22}N_6O_3$	/	/				
6.4733	351.1231 $[M-H]^-$	352.1309	Diethyl 2,2'-(2'-hydroxy-4',5-dioxo-2,4',5,5'-tetrahydro-1H,1'H-3,3'-bipyrrole-1,1'-diyl) diacetate	$C_{16}H_{20}N_2O_7$	/	/				

Abbraviations: FR: *Ficus racemosa* Linn., CM: *Clerodendrum petasites* (Lour.) S. Moore, HP: *Harrisonia perforata* (Blanco) Merr., TT: *Tiliacora triandra* (Colebr.) Diels. and CP: *Capparis micracantha* DC.

DISCUSSION

The present study, UHPLC-Q-TOF was used as a tool for isolation and identification of chemical constituents from HRF and its five herbal components. Untargeted screening method has been developed for identification of the possible chemical constituents of HRF and its five herbal components which is applicable for another complex herbal formula. The chemical compounds were identified by comparison of empirical molecular formula with online database, isotope pattern matching, and fragmentation pattern matching. According to the results, some peak intensities which were found in HRF are the same as those found in all or some herbal components too, which represented these peaks of HRF which were from its herbal components. On the other hand, some peak intensity was only established in HRF. It is possibly that the new compounds were produced after these herbal components were mixed. Moreover, some chemical constituents of the five herbal components were presented in a tiny concentration. When these herbal components were mixed in equal weight, the concentrations of chemical constituents were diluted. There was a possibility that some chemical constituents which were found in some or all five herbal components could not be found in HRF. Moreover, the chemical change of the constituents in HRF could be caused by chemical interaction of each herb during extraction processes.[26] This method was developed for rapid screening of the possible chemical constituents. However, the classification method which compares with reference standard should be done after screening.

CONCLUSION

This study has demonstrated a convenient, rapid screening, high-throughput and reliable UHPLC-Q-TOF method for analysis of chemical profiles. Under this optimization method, nineteen chemical constituents including phenolic and flavonoids were characterized in both positive and negative ESI mode. In addition, as shown in the PCA Score plot of positive and negative ESI modes, the chemical constituents of HRF were similar to those found in *Ficus racemosa* Linn. and *Capparis micracantha* DC. Hence, this work generated the new scientific data for HRF and its five herbal components to support the use of HRF and further study on identified compounds in each herbal component.

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Conflict of Interest: The authors declare no conflict of interest.

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