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Separation and Analysis of Seven Kind Compounds in Three Medicine Herbs Extraction by RP-HPLC

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Saururus chinensis, *Polygalae radix* and *Curcuma longa* are widely known as traditional oriental medicinal herbs with various pharmacological activities. These three herbs were efficiently identified with 60 % aqueous ethanol and then successively partitioned with hexane, dichloromethane, ethyl acetate, normal butyl alcohol and water to investigate the seven compounds rutin, quercitrin, quercetin, onjisaponin B, *bis*demethoxycurcumin, demethoxycurcumin and curcumin. The samples of these medicine herbs were simultaneously separated and analyzed by commercial C_{18} reverse phase high-performance liquid chromatography using the combination of water/trifluoroacetic acid and acetonitrile as the mobile phase in the isocratic and gradient elution mode. The analysis conditions were as follows: flow rate 1 mL/min, injection volume 10 µL and column oven temperature 40 °C at 200-400 nm wavelengths. The yield (%) and total extraction amount (mg) of *n*-BuOH (11.88-18.80 %, 1.2-9.4 mg) were higher than those of the other partition solvents hexane, dichloromethane and ethyl acetate. Also, the optimum mobile phase was simultaneously separated and analyzed. These results suggest that the extracts of these three medicinal herbs can be good therapeutic materials for anti-oxidant and anti-angiogenesis activities in the treatment of obesity.

Keywords: Analysis, HPLC, Optimization, Purification, Separation.

INTRODUCTION

The traditional medicines herbs have been used for thousands of years in many Asian countries[1] and have attracted interest and acceptance in many countries because of their few side effects, affordability and local availability[1,2]. Among them, *Saururus chinensis*, *Polygalae radix* and *Curcuma longa* are widely known as traditional oriental medicine herbs with various pharmacological activities[3-5]. They are perennial herbs that grow in the valleys and plains of Korea, China and Japan[6-8]. They have been reported to have a wide range of beneficial biological properties, including antitumor, antiinflammatory, anti-oxidant, anti-angiogenic activities for conditions such as beriberi, pneumonia, jaundice, as well as cognitive improving and potential antipsychotic efficacy[9-11], with low toxicity. Their pharmacological activities have been attributed mainly to related compounds. The main chemical constituents of *S. chinensis* are alkaloids, essential oils, flavonoids, lignans, neolignans, quercitrin, quercetin, rutin and tannin[12], those of *P. radix* are xanthone, xanthone C-glycosides, triterpene saponins, sucrose esters and oligosaccharide esters[13] and those of *C. longa* are rhizomes, which has 4-8 % volatile and 3-5 % curcuminoids[14].

Therefore, the seven compounds rutin, quercitrin, quercetin, onjisaponin B, *bis*demethoxycurcumin (BDMC), demethoxycurcumin (DMC) and curcumin in the three medicine herbs were analyzed quantitatively and qualitatively to determine the quality of the natural substance material or of its processed products[1]. Especially, many studies have been done on the analysis, identification, isolation, extraction and purification of medicine herbs[2]. In these studies, UHPLC, MS/MS, PTLC, LC-ELSD and NMR methods have been used to analyze and detect the chemical constituents of extracted samples and their metabolites[15-17]. In this study, the seven compounds rutin, quercitrin, quercetin, onjisaponin B, *bis*-demethoxycurcumin, demethoxycurcumin, curcumin were separated from the medicinal herbs *S. chinensis*, *P. radix* and *C. longa* and analyzed simultaneously. In addition, the effect of the partition solvents composition on the total extraction yield was studied. Also, the optimum mobile phase conditions were experimentally determined.

EXPERIMENTAL

The seven kind compounds rutin; (NPbank, Korea), quercitrin, onjisaponin B; (Chemfaces, China), quercetin, *bis*-demethoxycurcumin (BDMC), demethoxycurcumin (DMC),

curcumin; (Sigma, USA). The purity of the standard compounds determined using HPLC were higher than > 98 %. All the oriental medicinal herbs (the dried *S. chinensis*, *P. radix* and *C. longa*) were purchased from yeongcheon traditional herbal market (Gyeongsangbuk-do, Yeongcheon, Korea). The HPLC grade methanol (MeOH), acetonitrile (ACN) were purchased from J.T. Baker (Philipsburg, NJ, USA) and trifluoroacetic acid (TFA) was purchased from Sigma-Aldrich (Louis, MO, USA). The hexane, dichloromethane (DCM), ethyl acetate (EA) and normal butyl alcohol (*n*-BuOH) were purchased from daejung chemical (Gyeonggi-do, Shiheung, Korea). The triple distilled water was filtered with a 0.45 μm membrane filter (Advantec, Tokyo, Japan) before analysis. The chemical structures of seven kind compounds were shown in Fig. 1.

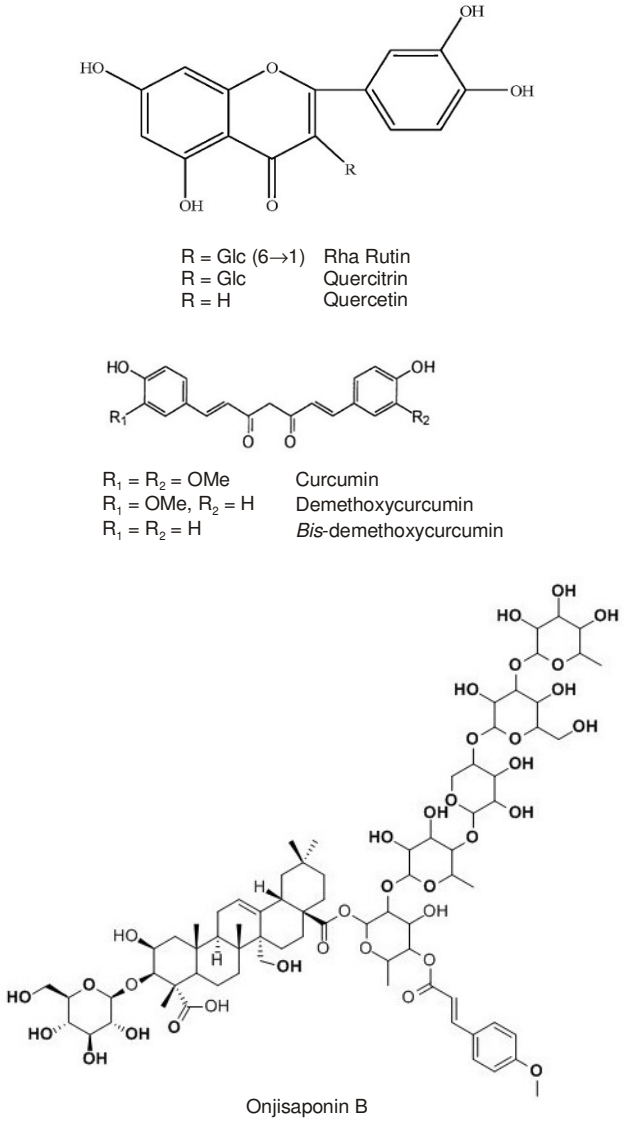

Fig. 1. Chemical structure of seven kind compounds in three medicine herbs

Preparation of standard: The solution of the standard samples were prepared by dissolving 2 mg of the standard chemical rutin, quercitrin, quercetin, onjisaponin B, *bis*-demethoxycurcumin (BDMC), demethoxycurcumin (DMC) and curcumin were prepared by dissolving 2 mg in a 10 mL MeOH solution (200 ppm).

Solvent extraction: All voucher specimens were deposited in the herbal bank of KM-based herbal drug development group, Korea institute of oriental medicine. 60 g dry samples from the powders of the three medicinal herbs (*S. chinensis*, *P. radix* and *C. longa*) containing were loaded in extraction system (first: 10 time volume) and (second: 5 time volume) of 60 % aqueous ethanol solution with reflux extraction, Following extraction, the extracted by heating for extraction time first: 3 h and second: 2 h at 70 °C, respectively. Then, the solution was filtered out using standard testing sieves (380 mesh), then the solvent was evaporated (concentration control: 20-30 brix) and then extract was freeze-dried and maintained in desiccators at 4 °C prior to use.

HPLC condition: The experiments were performed with a Dionex HPLC system equipped with an ultimate 3000 pump, ultimate diode array detector (DAD), injector 10 μL sample loop (Dionex, ID × L 0.18 × 550 mm Viper 550 mm USA) and chromeleon data acquisition system (Dionex version 7). Sufficient time was allowed for the stabilization of the C_{18} column and detector signal after each injection. The adjustable experimental variables were the conditions of gradient and isocratic modes mobile phase compositions. The chromatographic columns used in this experiment are commercially available, obtained from RS-tech, RP-column (0.46 mm × 25 cm, 5 μm, C_{18}, Korea). The injection volume was 10 μL and the flow rate of the mobile phase was 1 mL/min. The wavelength of the UV detector were 200-400 nm (fixed at 210, 254, 280 and 320 nm). The mobile phase solvents were A (water/trifluoroacetic acid = 99.9/0.1, v/v %) and solvent B (acetonitrile = 100, v/v %). First: the run time was 70 min and linear gradient method was applied mobile phase condition (solvent A: 0 min; 90, 60 min; 30, 70 min; 30 and B: 0 min; 10, 60 min; 70, 70 min; 70 %), Second: the run time was 60 min and isocratic method were applied variation mobile phase condition (solvent A: 80, 70, 60 and 50 % and B: 20, 30, 40 and 50 %), Third: the run time was 1 h and using the best separation and analysis conditions (solvent (B) % : 0 min; 12, 5 min; 15, 7 min; 18,10 min; 20, 13 min; 23, 15 min; 25, 17 min; 27, 20 min; 30, 23 min; 33, 25 min; 35, 27 min; 37, 30 min; 40, 60 min; 40) (Table-1).

RESULTS AND DISCUSSION

In this study, the seven compounds #1 rutin, #2 quercitrin, #3 quercetin, #4 onjisaponin B, #5 *bis*demethoxycurcumin (BDMC), #6 demethoxycurcumin (DMC) and #7 curcumin were separated from the three herbs (*S. chinensis*, *P. radix* and *C. longa*) and analyzed using reverse phase high-performance liquid chromatography (RP-HPLC). This procedure were optimized of simultaneous analysis condition, using different mobile phase conditions, 60 % aqueous ethanol and 3 h extraction time. The RP-HPLC method were used to qualitatively evaluate the extracts of two concentrations 10 and 50 mg/mL. Fig. 2 shows the chromatogram with linear gradient elution. Here, the 60 % aqueous ethanol could be extracted the more polar materials than the pure ethanol. The compounds were identified by comparing their retention times and UV spectra with those of the markers. The maximum UV absorption wavelength of 320 nm was applied. After, a standard

TABLE-1
ANALYSIS CONDITION OF GRADIENT AND ISOCRATIC ELUTION MODE WITH RP-HPLC

Instrument	Condition		
Reverse phase-column	RS-Tech optimapak C18 (4.6 × 250 mm, 5 µm)		
Oven temp. (°C)	40		
Flow rate (mL/min)	10		
Mobile phase (%)	A : 0.1 % trifluoroacetic acid in water, B : acetonitrile (ACN)		
UV Absorbance (nm)	200-400, (210, 25, 280 and 320)		
Time (min)	Solvent composition		
	0.1 % trifluoroacetic acid in water (A) %	Acetonitrile (B) %	
Linear gradient elution Run time : 70	90	10	(Fig. 2)
	30	70	
	30	70	
Isocratic elution Run time : 60	80	20 (a, b)	(Fig. 3)
	60	40 (a, b)	
Step gradient elution Run time : 60	(B) % : 0 min; 12, 5 min; 15, 7 min; 18, 10 min; 20, 13 min; 23, 15 min; 25, 17 min; 27, 20 min; 30, 23 min; 33, 25 min; 35, 27 min; 37, 30 min; 40, 60 min; 40. (61-75 min; Initial condition)		(Fig. 5)

solution of the seven compounds was qualitatively evaluated by HPLC. The result, the retention times of the seven compounds, were as follows: rutin: t_R 15.237 min, quercitrin: t_R 18.707 min, quercetin: t_R 25.887 min, onjisaponin B: t_R 37.563 min, *bis*demethoxycurcumin: t_R 43.270 min, DMC: t_R 44.543 min and curcumin t_R 45.817 min. However, quercetin (quercetin in *S. chinensis* was reported) was not detected in the 60 % aqueous ethanol extract sample[18]. Sample quality was influenced by the cultivation method according to the area. Fig. 3 shows the chromatogram of the seven compounds in each partition dichloromethane, ethyl acetate and *n*-BuOH phase. And each fraction by the extraction yield was measured. The mobile phase (A 80 : B 20 %) were extracted of rutin in *n*-BuOH phase and quercetin in ethyl acetate phase. Also, the mobile phase (A 60 : B 40 %) were extracted of onjisaponin B in *n*-BuOH phase and the *bis*demethoxycurcumin, demethoxycurcumin and curcumin in dichloromethane phase. Here, the quercetin could not be detected (Fig. 2 showed the same result). These, the amount of quercitrin and onjisaponin B compound in ethyl acetate and *n*-BuOH phase were remarkably higher, respectively. The seven compounds were characterized by comparing the HPLC UV-DAD maximum absorption peaks of the samples with those of the standards (Fig. 4). The seven kind compounds had strong absorption peaks near 210 and 320 nm. But, the *bis*demethoxycurcumin, demethoxycurcumin, curcumin in turmeric are well known to have strong absorption peaks at 400-425 nm[19]. Table-2 shows the data from the retention time (min) and peak area (mAU) analysis of the seven compounds with the mobile phase composition (%) for sample concentrations 10 and 50 mg/mL. When the column temperature was 40 °C, the mobile phase A compositions were 80, 70, 60 and 50 % and the mobile phase B compositions were 20, 30, 40 and 50 %. The result (by the sample 10 mg/mL), the increase in the mobile phase composition (A 80, 70 %) of the polar solvent (water) were confirmed in rutin t_R: 7.517, 3.473 min and in quercitrin t_R: 14.220, 4.677 min (here, did not detected quercetin). On the other hand, the decrease in the mobile phase composition (B 40, 50 %) of the nonpolar solvent (acetonitrile) was confirmed in onjisaponin B t_R: 12.627 min, 50 % (did not detected onjisaponin B) and *bis*demethoxycurcumin t_R: 27.387, 10.050 min, demethoxycurcumin t_R: 31.833, 11.343 min and curcumin t_R: 36.957, 12.830 min. Here, the sample 50 mg/mL was the shown same patterns with retention time and target compound detection. Table-3 shows

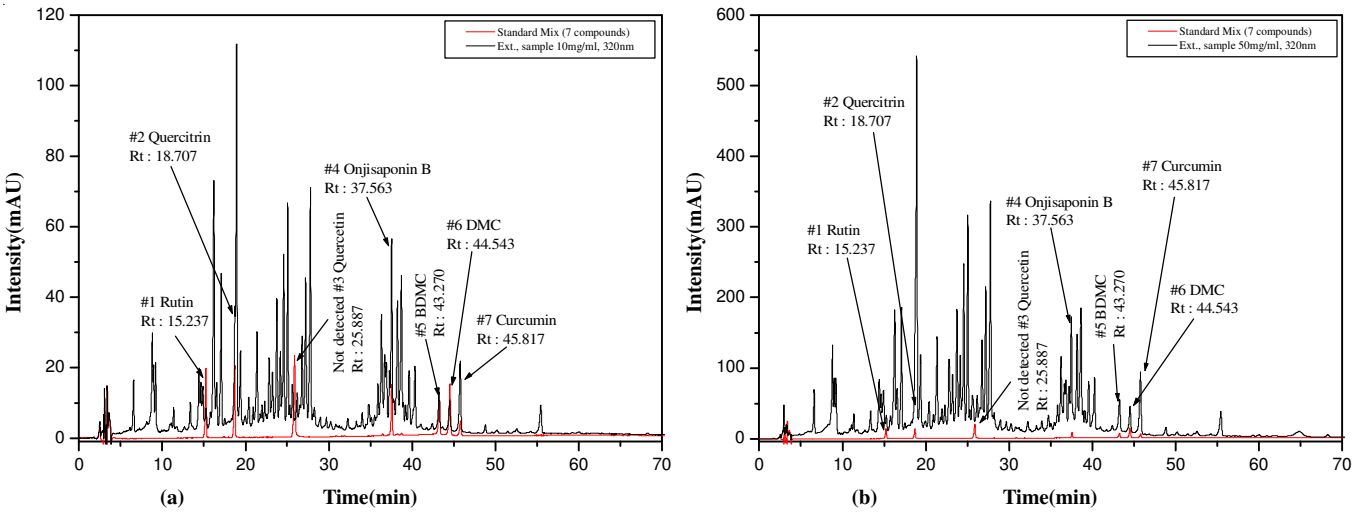

Fig. 2. Qualitative analysis of seven kind compounds in three medicine herbs extraction by RP-HPLC (sample concentration (a): 10 mg/mL, (b): 50 mg/mL, standard: 200 ppm, UV absorbance: 320 nm)

TABLE-2
ANALYSIS OF SEVEN KIND COMPOUNDS ACCORDING TO THE ISOCRATIC ELUTION

Mobile phase		Compounds name	t_R (min)	Sample concentration (mg/mL)	Average peak area (mAU)	SD	RSD (%)
H_2O (A)	CH_3CN (B)						
		Rutin #1	7.517		0.53	0.08	15.68
80	20	Quercitrin #2	14.220		3.23	0.12	3.59
		Quercetin #3	45.010		-	-	-
		Rutin #1	3.473		3.30	0.26	7.83
70	30	Quercitrin #2	4.677		2.68	0.01	0.28
		Quercetin #3	11.150		-	-	-
		Onjisaponin B #4	12.627	10	6.46	1.01	15.62
		BDMC #5	27.387		2.54	0.21	8.10
60	40	DMC #6	31.833		1.65	0.09	5.53
		Curcumin #7	36.957		3.72	0.34	9.14
		BDMC #5	10.050		2.40	0.25	10.49
50	50	DMC #6	11.343		2.11	0.23	10.95
		Curcumin #7	12.830		4.78	0.40	8.32
		Rutin #1	7.533		2.2	0.24	10.95
80	20	Quercitrin #2	14.247		14.70	0.13	0.87
		Quercetin #3	45.010		-	-	-
		Rutin #1	3.473		11.97	0.36	2.99
70	30	Quercitrin #2	4.673		11.58	0.09	0.78
		Quercetin #3	11.150		-	-	-
		Onjisaponin B #4	12.820	50	16.16	1.06	6.56
		BDMC #5	27.173		8.69	0.39	4.53
60	40	DMC #6	31.643		8.86	0.07	0.75
		Curcumin #7	36.813		17.42	0.57	3.29
		BDMC #5	10.007		10.10	0.78	7.75
50	50	DMC #6	11.290		9.21	0.97	10.51
		Curcumin #7	12.777		18.48	2.05	11.11

N.D (-) : not detected

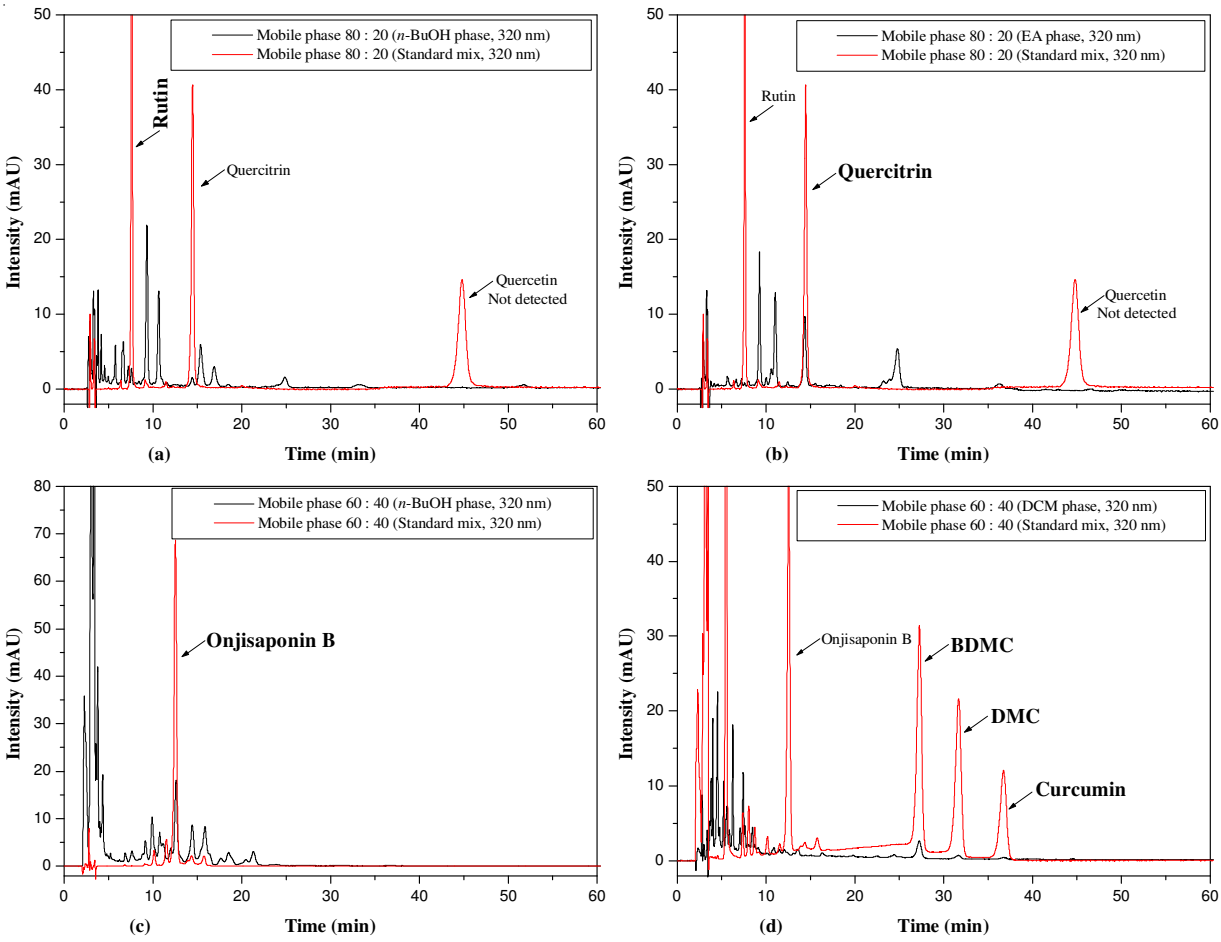

Fig. 3. Comparison behaviour of seven kind compounds in each partition extraction by RP-HPLC ((a): mobile phase; 80 : 20, *n*-BuOH phase; rutin, (b): mobile phase; 80 : 20, EA phase; quercitrin, (c): mobile phase; 60 : 40, *n*-BuOH phase; onjisaponnin B, (d): mobile phase; 60 : 40, DCM phase; BDMC, DMC and curcumin, UV absorbance: 320 nm, dry sample concentration: 1 mg/mL))

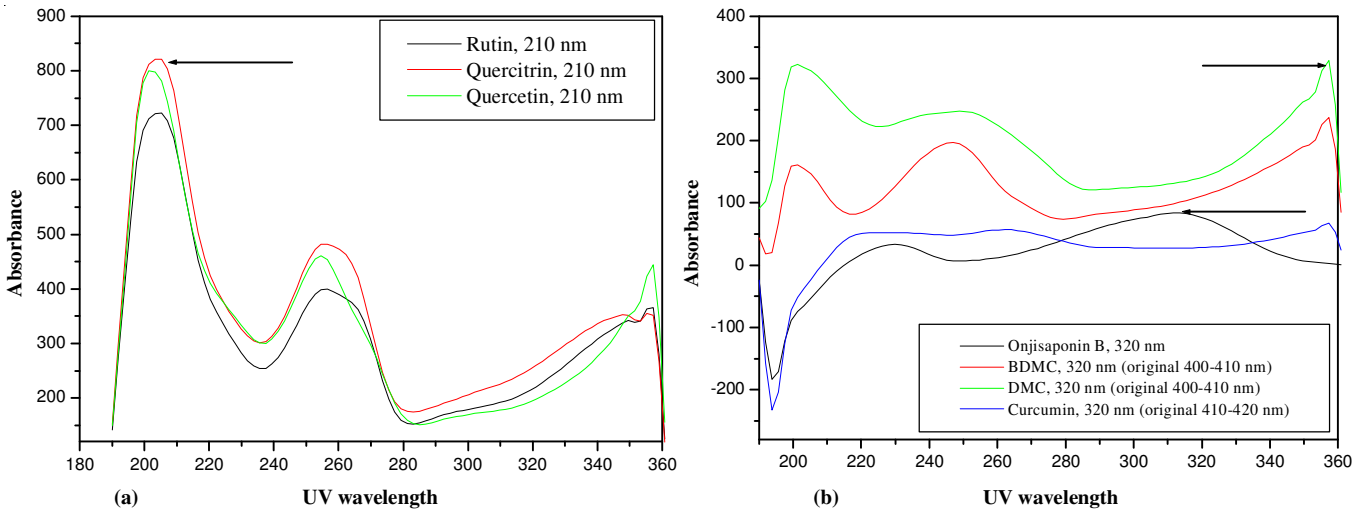

Fig. 4. HPLC-DAD UV Spectra of seven kind compounds

the extraction amount (mg) and yield (%) from each partition fraction sample. Four fraction organic solvents were used: hexane, dichloromethane (DCM), ethyl acetate (EA) and normal butyl alcohol (*n*-BuOH). This results were obtained the yield 7.00-9.90 % to 1-3.5 mg in hexane, yield 4.80-7.92 % to 0.8-3.5 mg in dichloromethane, yield 6.93-7.00 to 0.7-3.5 mg in ethyl acetate. Also, the highest yield and the total extract amount 11.88-18.80 % to 1.2-9.4 mg were obtained with *n*-BuOH. In general, the selection of extract solvent for extraction is very important. A polar solvent makes a soluble polar compound and thus, the extraction yield will be higher in that particular solvent. The HPLC analysis conditions for best separation and quantification of the seven compounds were successfully established by changing the solvent compositions of the mobile phase (Fig. 5)[20].

Conclusion

The simultaneous analysis of seven kind components rutin, quercitrin, quercetin, onjisaponin B, *bis*demethoxycurcumin, demethoxycurcumin and curcumin in three kind of medicinal herbs mixture (*S. chinensis*, *P. radix* and *C. longa*) using high-performance liquid chromatography (RP-HPLC).

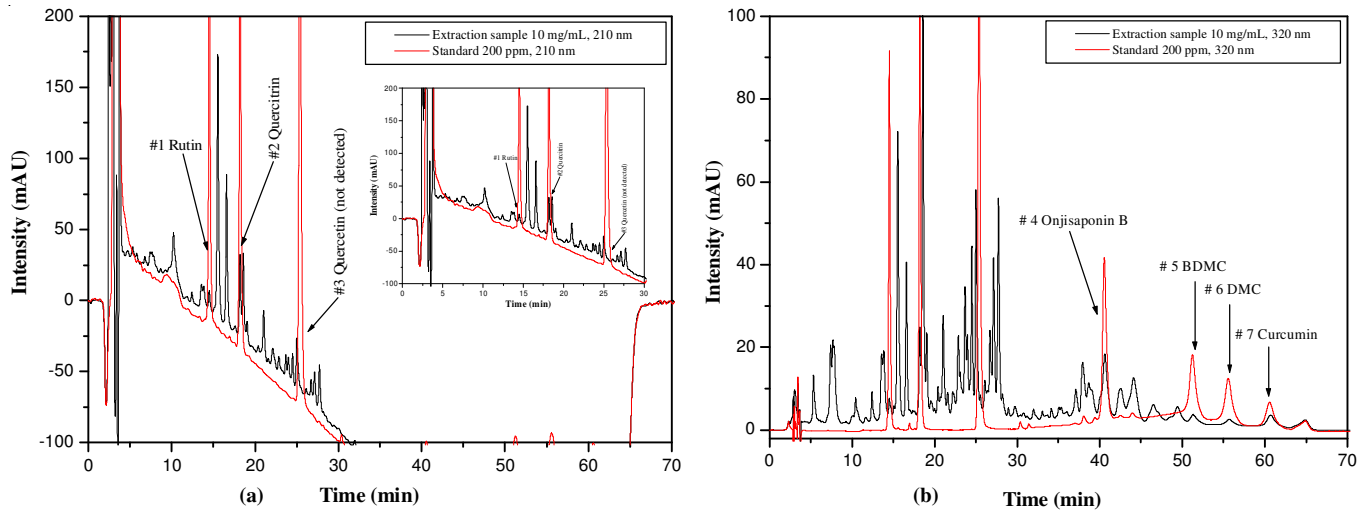

Fig. 5. Optimization analysis and separation of seven kind compounds in medicine herbs extraction by RP-HPLC (sample concentration: 10 mg/mL, standard: 200 ppm, UV absorbance: 210 and 320 nm)

TABLE-3
EXTRACT AMOUNT AND YIELD IN PARTITION COLLECTION SAMPLE

Partition solvent (× 3 time)	Partition loading amount (mg/mL)	Yield (%)	Total extraction amount (mg)
Hexane	10	9.90	1.0
Dichloromethane (DCM)		7.92	0.8
Ethyl acetate (EA)		6.93	0.7
n-Butyl alcohol (*n*-BuOH)		11.88	1.2
Hexane	30	7.00	3.5
Dichloromethane (DCM)		4.80	2.4
Ethyl acetate (EA)		7.00	3.5
n-Butyl alcohol (*n*-BuOH)		18.80	9.4

The efficiently identification of 60 % aqueous ethanol and then successively partitioned with hexane, dichloromethane, ethyl acetate, *n*-butyl alcohol and water in order to investigate the seven kind compounds. The results, the yield (%) and total extraction amount (mg) with the *n*-BuOH (11.88-18.80 % to 1.2-9.4 mg) were higher than any partition solvent. Also, the optimum mobile phase could be simultaneously separated and analyzed. Thus, the optimum operating conditions were experimentally determined. These results will be used to establish a database for the investigation of the constituents of natural products.

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