Chapter 1

Quantitative analysis of osteoprotective markers in 914

1.1. Experimental methods

1.1.1. Chemicals and reagents

The reference standards quercetin-6-C-β-D-glucopyranoside (K012), (2S,3S)-(+)-3'4'5,7-tetrahydroxydihydroflavonol-6-C- β -D-glucopyranoside (K058), naringenin-6-C-β-D-glucopyranoside (K068) and (2S,3S)-(+)-4'5,7-trihydroxydihydroflavonol-6-C- β -D-glucopyranoside (K100) (Fig-1.1) were obtained from MPC division, Central Drug Research Institute (C.D.R.I). Ononin (Fig-1.1) used as an internal standard (IS) was procured from Indofine Chemicals, New Jersey, U.S.A. HPLC grade methanol and analytical grade glacial acetic acid were purchased from LobaChemie Pvt. Ltd., Mumbai, India and S. D. Finechem limited, Mumbai, India respectively. Purified water was prepared in our division from Millipore Milli-Q system.

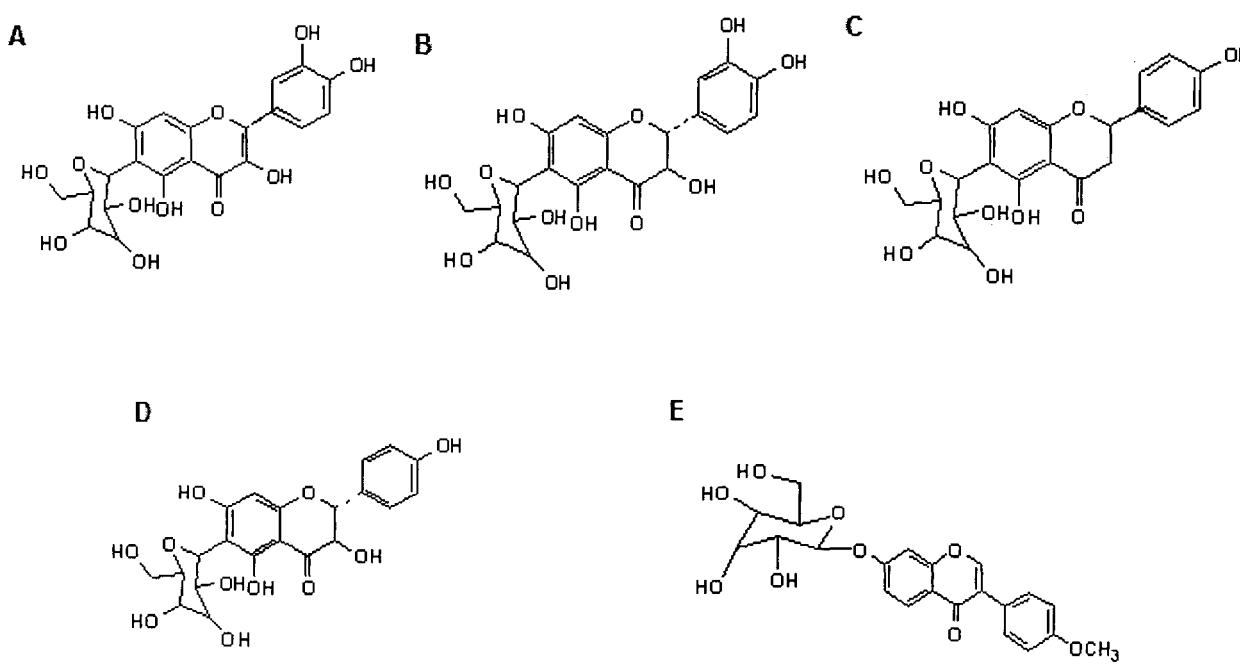

Figure-1.1: Chemical structure of **(A)** Quercetin-6-C-β-D-glucopyranoside (K012), **(B)** (2S,3S)-(+)-3'4'5,7-tetrahydroxydihydroflavonol-6-C-β-D-glucopyranoside (K058), **(C)** Naringenin-6-C-β-D-glucopyranoside (K068) **(D)** (2S, 3S) -(+)-4'5,7-trihydroxydihydroflavonol-6-C- β-D-glucopyranoside (K100) **(E)** Ononin

1.1.2. Herbal preparations

The aqueous fraction 914/C008 (AFB) and butanolic fraction 914/F009 (BFB) were extracted from the stem bark of *Ulmus wallichiana*. The aqueous fraction (AFT), ethanol fraction (EFT) and butanolic fraction (BFT) were obtained from the twig of *Ulmus wallichiana*. All these herbal preparations were extracted in MPC division, CDRI.

1.1.3. Preparations of stock and working solutions of standards

4mg each of K012, K058, K068 and K100 was dissolved separately in 1ml of methanol to get a primary stock solution of 4mg/mL. A composite working solution of K012, K058, K068 and K100 at a concentration of 500µg/mL each was prepared by mixing 500µl of individual stock solutions (4mg/mL) in 2mL of methanol. This working solution was further serially diluted to get working solutions in the concentration ranging from 250 to 3.9µg/mL in methanol. These working solutions were used to prepare analytical standards (AS). A 1mg/mL of IS stock solution was prepared by dissolving 1mg of Ononin in 1ml of methanol. From it, IS working solution of concentration 500µg/mL was prepared by diluting with methanol.

A composite working solution of K012, K058, K068 and K100 at the concentration of 400µg/mL was also prepared in methanol from their stock solutions (4mg/mL). Working solutions of 200, 50 and 10 µg/mL were prepared by stepwise dilution of 400µg/mL working solution. These working solutions were used to prepare quality control (QC) samples. All these working solutions were stored at 4^0C.

1.1.4. Preparation of analytical standards and quality control samples

Analytical standards (AS) of concentration ranging from 0.39µg/mL to 25µg/mL were prepared by spiking 25µl of the corresponding working solution (3.9 to 250 µg/mL) in 210µl of dilution solvent, methanol : 0.3% acetic acid (10:90, v/v). In this 15µl of IS working stock was spiked to get the concentration of 30µg/mL. 50µl of these standards were injected for analysis by HPLC-PDA. The quality control (QC) samples at three different levels of low (QCL-1µg/mL), medium (QCM-5µg/mL) and high (QCH-20µg/mL) were prepared by

spiking 25µl of QC working solutions 10 µg/mL, 50µg/mL, and 200µg/mL respectively in 210µl of dilution solvent. 15µl of 500µg/mL IS was spiked in all QCs (Table-1.1).

Table-1.1: Preparation of analytical standards (AS) and quality controls (QC)

Concentration of WS[a] (µg/mL)	Volume of WS + DS[b]	AS/QC concentration (µg/mL)
3.9	25µL + 210µL	AS 0.39
7.8	25µL + 210µL	AS 0.78
15.6	25µL + 210µL	AS 1.56
31.25	25µL + 210µL	AS 3.12
62.5	25µL + 210µL	AS 6.25
125	25µL + 210µL	AS 12.5
250	25µL + 210µL	AS 25
10	25µL + 210µL	QC 1
50	25µL + 210µL	QC 5
200	25µL + 210µL	QC 20
To each AS and QC, 15 µL of IS (500 µg/mL) was added		

Note: a - Working Solution of mix of K012, K058, K068 and K100,
b - Dilution Solvent (90:10 of methanol : 0.3% acetic acid)

1.1.5. Chromatographic conditions

The analyses were carried out using Shimadzu LC 20AD UFLC pumps, a SIL-HTc controller with autosampler, CTO-20AC column oven and SPD-M20A prominence PDA detector. Phenomenex C_{18} column (250 x 4.6mm, 5µ) coupled with a Phenomenex guard column was used for chromatographic separation. The chromatographic data so obtained were recorded and analyzed by LC solution ver 1.22 SP1software, Shimadzu. The gradient elution of 65min with a mobile phase solvent A (methanol) and solvent B (0.3% acetic acid) at a flow rate of 1.5ml/min was used for optimum separation. The gradient program employed was as follows: initial convex (10) gradient from 90% to 83% B (0-20min) followed by a linear gradient, 83% to 50% B (20-50min), isocratic at 50% B (50-52min), then 50% to 90% B (52-53min), finally isocratic at 90% B till 65min (Table-1.2). The

injection volume was 50µl. The detection wavelength was set at 260nm for quantitative analysis of K012 and 290nm for other markers K058, K068 and K100.

Table-1.2: Gradient condition for the separation of markers of 914

Time (min)	A[a] (%)	B[b] (%)	B curve
0.1	10	90	10
20.0	17	83	10[c]
50.0	50	50	--
52.0	50	50	--
53.0	10	90	--
65.0	10	90	--

Note: a- methanol, b - 0.3% acetic acid,
c - B curve = from 0 to 20.01min onwards

1.1.6. Validation of HPLC-PDA method

The developed HPLC-PDA method was validated to evaluate the linearity range, limit of detection (LOD), limit of quantitation (LOQ), precision and accuracy.

Linearity and sensitivity

To determine the linearity, analytical standards consisting of K012, K058, K068 and K100 were prepared in the concentration range from 0.39µg/mL to 25µg/mL. The analysis was performed on three days and the calibration curve was derived for each analyte by plotting peak area ratio of each compound to IS versus concentration. The linearity was evaluated by linear regression analysis calculated by least square regression method so that regression coefficient (r) value obtained was greater than 0.99.

For each analyte, the limit of detection (LOD) and lower limit of quantitation (LLOQ) under the present chromatographic conditions were evaluated from signal to noise ratio (S/N). For LOD and LLOQ, S/N should be greater than 3 and 10 respectively. This determines the sensitivity of the method.

Precision and accuracy

Intra-day and inter-day variations were used to assess the accuracy and precision of the established method. These variations were determined form the quality control samples; QCL (1µg/mL), QCM (5µg/mL) and QCH (20µg/mL). To evaluate intra-day variations five replicates of each QC were examined within a day, while for inter-day variations five replicates of each QC were analyzed for three consecutive days. Precision was calculated in terms of % C.V. using ANOVA, while for accuracy, % bias was calculated. The precision and accuracy limits considered for standards were ±15% except for LLOQ, for which ±20% of its nominal value is acceptable.

1.1.7. Sample preparation

Stock solutions of 1mg/mL each of herbal extracts were prepared separately by dissolving them in methanol. From these stock solutions, dilutions of 800 and 400µg/mL each of BFB (914/F009), AFB (914/C008), AFT, BFT and EFT were prepared separately in dilution solvent. 15µL of IS (500µg/mL) was spiked in all the dilutions to get the concentration of 30µg/mL of IS (Table-1.3).

Table-1.3: Dilution of herbal fractions

Concentration of HF[a] (mg/mL)	Volume of HF dried (µL)	Reconstitution volume of DS[b] (µL)	Final concentration of HF(µg/mL)
1	200	225	800
1	100	225	400
To each dilutions, 15 µL of IS (500 µg/mL) was added			

Note: a-Herbal fraction, b-Dilution solvent

1.2. Results and Discussion

1.2.1. Optimization of chromatographic conditions

The development of gradient method for simultaneous quantification of four markers was difficult due to presence of other unknown and related components in herbal extracts. Moreover, the two markers, K012 and K068 eluted very close to each other. For the marker K058, a closely eluting peak was obtained, which could be due to presence of isomer as an impurity. The resolving of this co-eluting peak was also difficult. The chromatographic

conditions were optimized to obtain chromatogram with best possible resolution of these adjacent peaks with symmetrical peak shape for all four analytes and IS in standard mix and herbal extracts. Various chromatographic conditions such as different columns (Discovery C_{18}, Zorbax C_{18}, Phenomenex C_{18}), mobile phase compositions, pH, flow rates and time program were investigated. Finally, an acceptable separation was achieved with gradient elution of 65min at the temperature of 30°C using Phenomenex C_{18} column with methanol and 0.3% acetic acid as a mobile phase delivered at a flow rate of 1.5ml/min. The optimum injection volume for analysis was 50µl.

Primarily, the identification of chromatographic peaks was done by comparing the retention time of peak and the spectrum of analyte in herbal extracts with that of reference standards under the same conditions. Additionally, the enhancement in the intensities of the identified peaks in the herbal extracts by spiking the mix reference standards in samples, gives further confirmation about their identification. The retention time for K012, K058, K068, K100 and IS was 38.78, 16.75, 36.40, 24.70 and 47.68 min respectively. Peak shape and retention time were consistent for all analytical runs. Fig-1.2 shows the representative chromatogram of mixed standard and all herbal extracts. The detection wavelength was chosen as 290nm for K058, K068 and K100 and 260nm for K012, at which the maximum absorption was observed (λ_{max}).

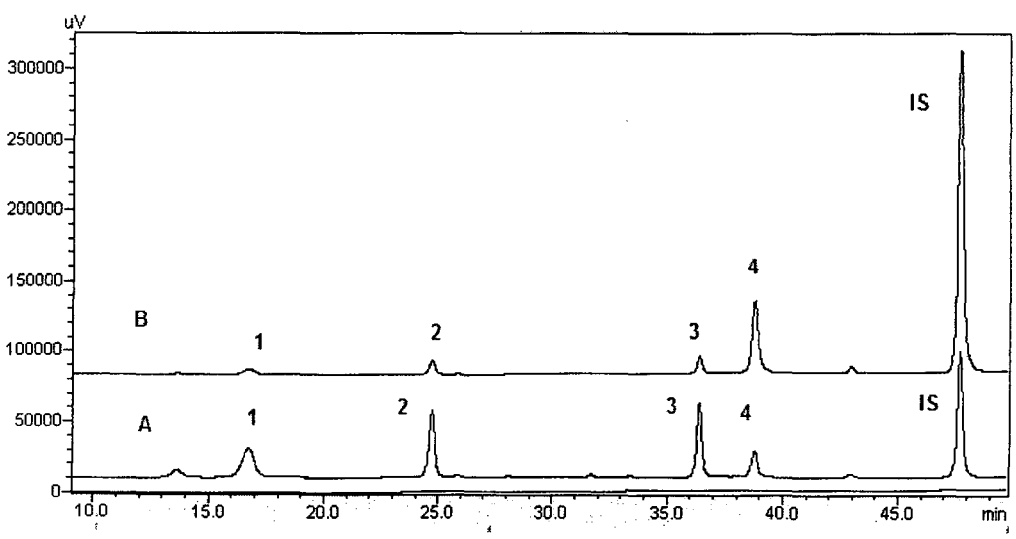

Figure-1.2: HPLC-PDA chromatogram of (A) reference standards at 290nm, (B) reference standards at 260nm overlaid on blank. (1) K058 (2) K100 (3) K068 and (4) K012

1.2.2. Validation of HPLC-PDA method

The HPLC-PDA method developed was validated for linearity, sensitivity, accuracy and precision. The calibration curves obtained for all four markers showed good linearity for the concentration range from 0.39µg/mL to 25µg/mL. The values of regression co-efficient (r) were greater than 0.999 for the calibration curves plotted for these markers on three different days (N=3) (Table-1.4). The LOD and LLOQ were 0.19µg/mL and 0.39µg/mL respectively for each marker. As shown in Table 1.5, the % bias and % C.V. values of QCs of four markers were less than 12% for inter-day and intra-day accuracy and precision. All the values obtained were within the acceptable limit of ±15%. Thus, the validated HPLC-PDA method was reliable for the simultaneous determination of K012, K058 K068 and K100.

Table-1.4: Validation data of HPLC-PDA method for 914 markers

Markers	t_R[a] (min)	LOD[b] (µg/mL)	LLOQ[c] (µg/mL)	Linearity (µg/mL)	Regression Coefficient (r)
K012	38.80	0.195	0.39	0.39-25	0.999±0.0011
K058	16.75	0.195	0.39	0.39-25	0.9998±0.0001
K068	36.40	0.195	0.39	0.39-25	0.9997±0.0001
K100	24.70	0.195	0.39	0.39-25	0.9999±0.0001

Note: a-Retention time, b- Limit of Detection, c- Lower Limit of Quantification

1.2.3. Application of the method

Quantification of marker components in herbal extracts

The validated HPLC-PDA method was successfully applied for the quantification of active markers K012, K058, K068 and K100 in different herbal extracts obtained from stem bark and twigs of *Ulmus wallichiana.* The content of four marker components in herbal extracts was determined by using regression equation obtained from calibration curves of each analyte. The results are summarized in Table-1.6.

Table-1.5: Intra-day and inter-day accuracy and precision for markers of 914

Markers	Quality control (µg/mL)	Accuracy (% Bias)		Precision (% C.V.)	
		Intra-day	Inter-day	Intra-day	Inter-day
K012	1	4.84	4.41	11.91	5.93
	5	-3.15	-4.55	1.89	10.32
	20	-4.33	-4.10	1.56	1.97
K058	1	0.03	0.64	3.99	5.88
	5	-3.78	-1.33	0.98	6.77
	20	1.67	1.40	1.48	1.81
K068	1	-0.59	2.90	8.31	5.45
	5	-9.26	-11.19	0.84	7.83
	20	-4.32	-4.10	0.49	1.07
K100	1	5.79	-0.29	8.29	9.44
	5	5.39	0.41	11.48	8.72
	20	-8.67	-8.43	2.82	3.95

Table-1.6: Percentage content of markers in herbal fractions of *Ulmus wallichiana*

Herbal Fractions	K012 (Mean±S.D.)	K058 (Mean±S.D.)	K068 (Mean±S.D.)	K100 (Mean±S.D.)
BFB (914/F009)	11.77±0.33	1.29±0.08	0.85±0.02	0.70±0.01
AFB (914/C008)	BLOQ	0.65±0.11	0.05±0.01	0.75±0.03
AFT	0.17±0.01	0.15±0.01	0.05±0.004	0.03±0.003
BFT	0.60±0.01	0.20±0.001	0.21±0.03	0.16±0.02
EFT	0.41±0.005	0.16±0.002	0.09±0.001	0.04±0.001

Note: BFB - Butanolic fraction of bark, AFB - Aqueous fraction of bark, AFT - Aqueous fraction of twigs, BFT - Butanolic fraction of twigs, EFT - Ethanolic fraction of twigs, BLOQ – Below limit of quantification

Comparative analysis between herbal extracts

The results in Table-1.6 clearly reveal that there is a considerable difference in percentage content of K012, K058, K068 and K100 among the herbal extracts obtained from *Ulmus wallichiana.* Comparatively, the amount of K012 was greatest (11.77±0.33) in butanolic

extract of bark (914/F009) than in other herbal fractions of *Ulmus wallichiana*. The content of K012 in aqueous extract of bark (914/C008) was found to be below the level of quantification (BLOQ). In twig extracts of *Ulmus wallichiana*, the percentage of K012 was less than 1%. Similarly, the content of markers K058 (1.29±0.08) and K068 (0.85±0.02) was also highest in 914/F009 in comparison to that of other herbal fractions.

The content of K100 was comparable in both 914/F009 (0.70±0.01) and 914/C008 (0.75±0.03) bark fractions. The fractions obtained from the twigs have lower level of K100, about 0.16±0.02% found in butanolic extract of twig while less than 0.05% was present in aqueous and ethanolic extracts of twig of *Ulmus wallichiana*. Overall, the level of all four markers was found to be more in butanolic extract (914/F009) of stem bark of *Ulmus wallichiana*. Among the twig extracts also, the percentage content of four markers was higher in BFT in comparison to other twig extracts.

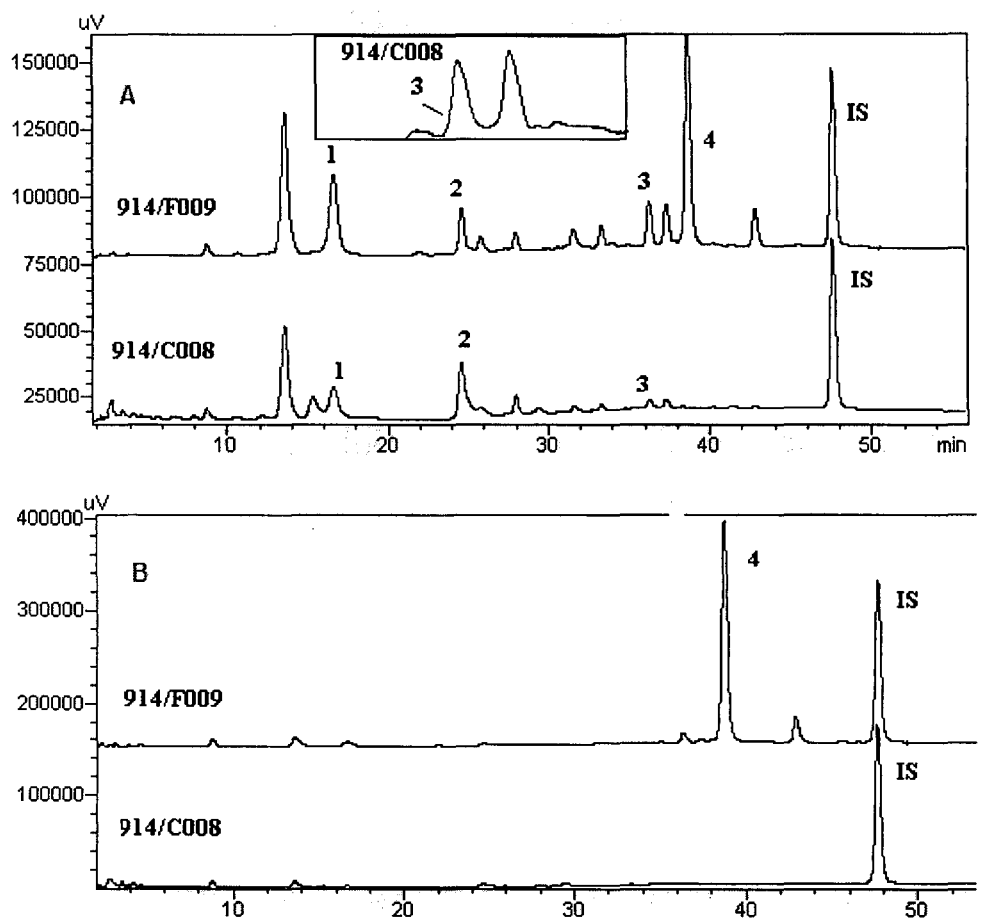

Figure-1.3: Overlay of HPLC-PDA chromatogram of 914/F009 and 914/C008, (A) at 290nm (B) at 260nm. (1) K058 (2) K100 (3) K068 and (4) K012

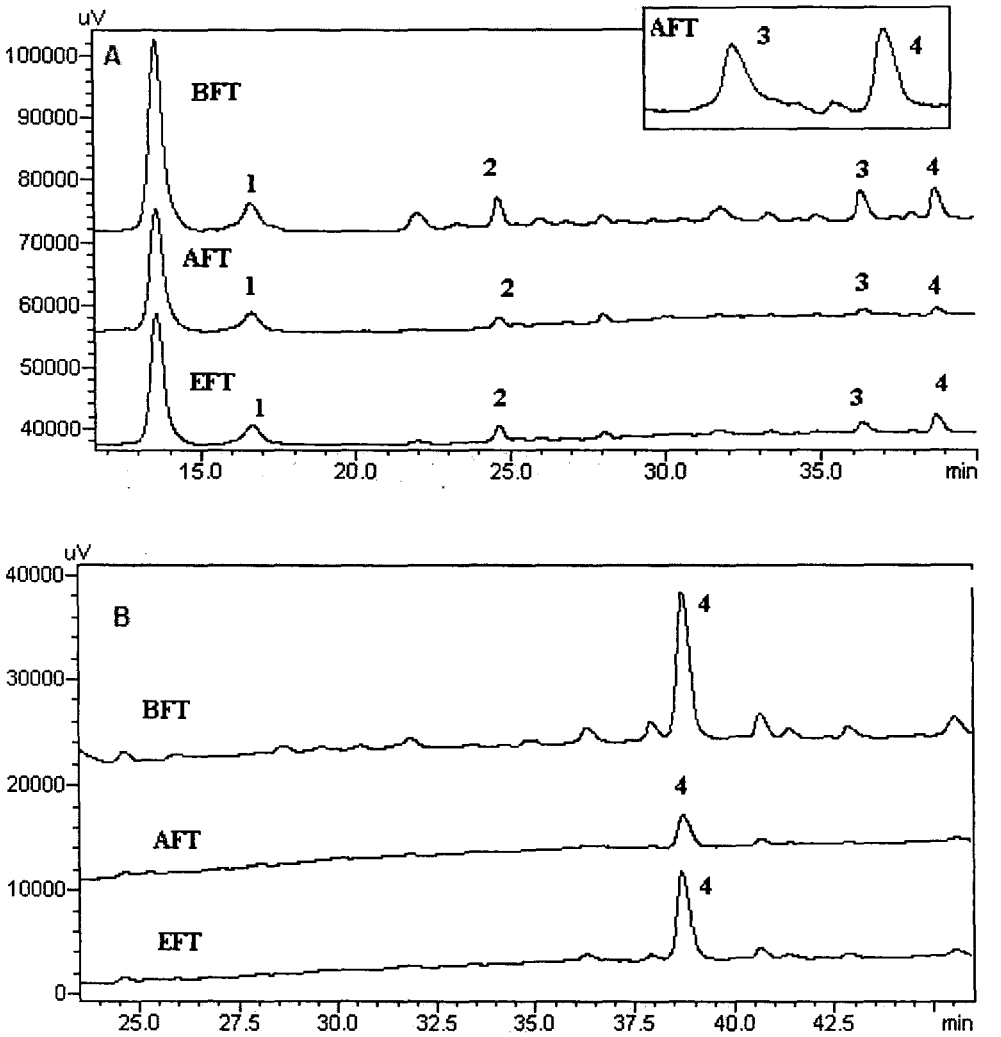

Figure-1.4: Overlay of HPLC-PDA chromatogram of BFT, AFT and EFT (A) at 290nm (B) at 260nm. (1) K058 (2) K100 (3) K068 and (4) K012

1.3. Conclusion

In this study, HPLC-PDA method has been developed and validated for the simultaneous analysis of quercetin-6-C-β-D-glucopyranoside (K012), (2S,3S)-(+)-3'4'5,7-tetrahydroxydihydroflavonol-6-C-β-D-glucopyranoside (K058), naringenin-6-C-β-D-glucopyranoside (K068) and (2S,3S)-(+)-4'5,7-trihydroxydihydroflavonol-6-C-β-D-glucopyranoside (K100). The validated HPLC-PDA method was selective, accurate and precise with % bias and % C.V. values within acceptable limits of ±15%. The LOD and LLOQ of the method were 0.19μg/mL and 0.39μg/mL respectively for each C-glycoside.

The linearity of the HPLC-PDA method ranged from 0.39μg/mL to 25μg/mL for all the four C-glycosides. The validated method was applied to determine the percentage content of these four active constituents in different herbal extracts obtained from *Ulmus wallichiana.* The percentage content of all four markers was found to be highest in the butanolic extract of stem bark while their level was very low in the aqueous extract. In herbal fractions obtained from the twig of *Ulmus wallichiania*, the level of each marker was less than 1%. Thus by applying the validated HPLC-PDA method, the variations in the content of four active osteogenic constituents in different herbal extracts of *Ulmus wallichiana* were evaluated.