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ORIGINAL ARTICLE

Simultaneous Detection and Quantification of Biomarker Glycosides in different Atriplex species using HPTLC

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ABSTRACT
A sensitive and accurate high-performance thin layer chromatography (HPTLC) method has been developed to determine the quantity of rutin and aloe emodin simultaneously in two different Atriplex species extracts. Rutin and Aloe emodin were separated on aluminum-backed silica gel 60 F254 plates with EtOAc: MeOH: H2O (30:5:4)(%, v/v) as mobile phase. Compact bands were obtained at Rf value of 0.33 ±0.04 and 0.43 ± 0.03 for rutin and aloe emodin respectively. The calibration plots were linear in the range of 100-800 and 100-700 ng/spot and the correlation coefficient of 0.9971 and 0.9989for rutin and aloe emodin respectively. Densitometric quantitation of rutin was performed at 358 nm, while aloe emodin at 262 nm. The developed HPTLC densitometric method was found cheap, selective, precise and accurate and can be used for routine analysis of two different Atriplex species extracts..
***Keywords**: Thymoquinone; HPTLC densitometry; ICH guidelines; Qualitative; Quantitative*

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INTRODUCTION

The genus *Atriplex* comprises about 200 species and belongs to subfamily Chenopodiaceae. *Atriplex farinosa* is a tall shrub of yellow white appearance with large, naked panicles, but leaf base cordate with long, obtuse auricles, fruit bracts entire, longer than broad, acute [1]. Some reports suggested the presence of naringin, naringenin 7-*O*-glucoside, isorhamnetin 3-*O*-rhamnosyl (1-6) glucopyranoside and isorhamnetin-7-*O*-glucopyranoside in *A. farinosa* [2].

In traditional medicine, a cocktail of minerals in *A. halimus* is used to benefit glycaemic control in diabetic patients [3]. Like other halophytes, it used in veterinary medicine to combat internal parasites [4].*A. halimus* produces the polyphenols and other bioactive substances which potentially useful for medicinal properties and as natural food preservation [5]. *A. confertifolia* has significant bioactivity against human breast cancer cell lines; the bioactivity of *A. confertifolia* extract on these cells was compared to a FDA-approved cancer drug; Onxol and an industry-standard leukocyte control cell line. Active portions of the extract were found primarily in the polar fractions of the plant. A dose-response curve of the extracts displayed significant cell death similar to Onxol[6].

Five compounds were isolated from this plant: quercetin-4′ -methoxy-7-glucorhamnoside (1), kaempferol -4′ -methoxy-3- glucorhamnoside (2), quercetin -6, 4′ -dimethoxy -3- glucorhamnoside (3), scopolin (4) and scopoletin (5)[7]. *A. farinosa and A. nummularia* (200 and 400 mg/kg) possesses a promising antihyperglycemic effect that is comparable with glibenclamide [8].

Several HPLC methods were developed for the quantitative analysis of rutin and aloe emodin separately. The HPTLC methodsreported have been used to quantify rutin and aloe emodin. To our knowledge, no report on simultaneous quantitative analysis of rutin [Figure 1] and aloe emodin [Figure 2] has been reported in different Atriplex species using HPTLC. In the present study we have proposed new validated simple HPTLC for the simultaneous determinationrutin and aloe emodin. The proposed method was validated as per ICH guidelines [9].

MATERIAL ANDMETHODS

Standards and chemicals

Standard Rutin and Aloe emodin were purchased from Sigma-Aldrich, St. Louis, MO, USA.All the solvents were of HPLC grade and other chemicals used were of analytical reagent (AR) grade.

Accurately weighed 10 mg of standard rutin and aloeemodin were dissolved in MeOH in a 100mL volumetric flask to gives concentration of 100µg/mL. These solutions were used as a reference solution (stock solution) for rutin and aloeemodin**.**

Plant materials

The whole plants of *Atriplex nummularia* and *Atriplex farinosa* were collected from the local area of Al-Kharj city, Kingdom of Saudi Arabia. The plants were identified by Dr. Mohammed Yusuf, Taxonomist at the Research Center of Medicinal, Aromatic and Poisonous plants, a voucher specimen was deposited in the Herbarium of the Department of Pharmacognosy, College of Pharmacy, Prince Sattam bin Abdulaziz University for future reference.

Extractions procedure

The dried ***whole plants*** (10 g) were extracted by percolation at room temperature with MeOH till exhaustion. The solvents were evaporated under reduced pressure and the residues were dissolved in methanol using 50 mL volumetric flask. These solutions were used as the test solutions in the TLC densitometric analysis.

Chromatographic conditions

HPTLC densitometric analysis was performed on 10 × 20 cm aluminium-backed plates coated with 0.2 mm layers of silica gel 60 F_{254} (E-Merck, Germany). Samples were applied to the TLC plates as 6 mm bands using a Camag Automatic TLC Sampler 4 (ATS4) sample applicator (Switzerland) fitted with a Camagmicrolitre syringe. A constant application rate of 150 nl/swas used. Linear ascending development of the plates to a distance of 80 mm was performed with ethyl acetate: methanol: water 30:5:4 (%, *v/v*) as mobile phase in a Camag Automatic Developing Chamber 2 (ADC2) previously saturated with mobile phase vapour for 30 min at 22 °C.

Method Validation

The proposed HPTLC method was validated according to the guidelines of international conference on harmonization (ICH) [9]for different parameters like linearity,accuracy, precision, LOD and LOQ and robustness [10, 11, 12].The linearity was checked 100-800 and 100-700 ng/spot for rutin and aloe**emodin** respectively, and concentrations were plotted against peak area.

Accuracy

Accuracy, as recovery, was determined by the standard addition method. Pre-analyzed samples of rutin and aloe emodin (300 ng/spot) were spiked with extra rutin and aloeemodin standard (0, 50, 100, and 150%) and the mixtures were reanalyzed. Percentage recovery and relative standard deviation (RSD, %) were calculated for each concentration level.

Precision

Precision was assessed by determination of repeatability and intermediate precision. Repeatability of sample was determined as intra-day variation whereas intermediate precision was determined by assessment of inter-day variation for analysis of rutin and aloe emodin at three different amounts (300, 400, and 500 ng/spot) in six riplicate.

Robustness

Robustness of the proposed TLC densitometric method was determined to evaluate the influence of small deliberate changes in the chromatographic conditions during determination of rutin and aloe emodin. Robustness was determined by changing the polarity of the mobile phase.

Limit of detection (LOD) and limit of quantification (LOQ)

Limit of detection (LOD) and limit of quantification (LOQ) were determined by standard deviation (SD) method. They were determined from the slope of the calibration (S) curve and SD of the blank sample using following equations:

$$\mathbf{LOD = 3.3\times SD / S}$$

LOQ = 10× SD /S

Specificity

Specificity of the proposed TLC densitometric method was confirmed by the R_f and spectra of the spot with that of the standards.

Quantification of Rutin and Aloe emodin in methanolic extract of *Atriplex nummularia* and *Atriplex farinosa*.

The test samples were applied and chromatograms were obtained under the same conditions as for analysis of standard rutin and aloeemodin. The area of the peak corresponding to the $R_f$value of rutin and aloeemodin standard were recorded and the amount present were calculated from the regression equation obtained from the calibration plot.

RESULTS AND DISCUSSION

Method development

The mobile phase's compositions were optimized to establish a suitable and accurate densitometric HPTLC method for simultaneous analysis of rutin and aloeemodin. The mobile phase composed of EtOAc: MeOH: H_2O (30:5:4)(%, *v*/*v*) resulted in a sharp, symmetrical, and well resolved peak at R_f value of 0.33 ± 0.04 and 0.43 ± 0.03for rutin and aloeemodin respectively. UV spectra measured for the peaks showed maximum absorbance at approximately 358 and 262 nm for rutin and aloeemodin respectively. (Figure 3 & 4).

Calibration curve

The calibration plot of peak area against amount of rutin and aloeemodin were linear in the range 100-800 ng/spot and 100-700 ng/spot. Linear regression data for the plot confirmed the good linear relationship (Table 1). The correlation coefficient (R^2) were 0.9971and 0.9989 for rutin and aloeemodin respectively which was highly significant ($P<0.05$). The linear regression equation was Y = 6.3679x + 789.44and Y = 6.3014x + 1195.1for rutin and aloe emodin respectively, where Y is response and X is amount of reference standards.

Method validation

Precision

The accuracy of the method, as recovery, was 98.28-99.25% and 98.82-99.35%, with RSD values in the range 0.53-1.18and 0.66-1.32 for rutin and aloe emodin respectively. These results indicated the accuracy of the method (Table 2). Results from determination of repeatability and intermediate precision, expressed as SD (%) are shown in Table 3. RSD was in the range 0.52-1.02and 0.62-1.06 for repeatability, 0.51–0.81 and 0.52-0.87 for intermediate precision for rutin and aloe emodin respectively. These low values indicated that the method is precise.

Robustness of the Method

Results of robustness are shown in Table 4. Low values of % RSD 0.66-0.90 and 0.71-0.92 for rutin and aloe emodin respectively were obtained after introducing small deliberate change into the densitometric TLC procedure proved the robustness of the proposed HPTLC method.

Limit of detection and quantification

LOD and LOQ of the proposed method were found to be 7.73 and 23.42 ng/spot, 6.62 and 20.05 ng/ml for rutin and aloe emodin respectively, which indicated that the proposed method can be used in wide range for detection and quantification of rutin and aloe emodin effectively.

Specificity

The peak purity of rutin and aloe emodin were assessed by comparing the overlaid spectra at peak start, peak apex and peak end position of the spot. The overlaid spectra of rutin and aloe emodin standards and methanolic extract of *Atriplex nummularia* and *Atriplex farinosa* were given in Figure 7 and 8.

Quantification of Rutin and Aloe emodin in methanolic extract of *Atriplex nummularia* and *Atriplex farinosa*

Rutin and Aloe emodin in methanolic extract of *Atriplex nummularia* and *Atriplex farinosa* by comparing their single spot at R_f = 0.33 and 0.43 with that of standard rutin and aloe emodin were given in figure 3 and 4. The amount of rutin and aloe emodin present in the methanolic extract of *Atriplex nummularia* [5] and *Atriplex farinose* [6]were found to be 0.89, 0.54 and 0.44, 0.37respectively.

Research outcome

The attempt here is to develop a novel HPTLC method which can be employed for the proper, identification and simultaneous quantification of rutin and aloe emodinin methanolic extract of *Atriplex nummularia* and *Atriplex farinosa*. The proposed method will be optimized in such a way to give maximum resolutions between the components, which are critical to avoid merging/overlapping components and makes the method superior to other existing methods. Further, the developed HPTLC

method was evaluated for different parameters as per the ICH guidelines and found accurate and reproducible, The method is suitable for the routine analysis of these components in different crude as well as herbal formulations.

Table I: Linear regression data for the calibration curve of rutin and aloe emodin(n=6)

Parameters	Rutin	Aloe emodin
Linearity range (ng/spot)	100-800	100-700
Regression equation	Y = 6.367x + 789.44	Y = 6.3014x + 1195.1
Correlation coefficient	0.9971	0.9989
Slope ± SD	6.367 ± 0.3367	6.3014 ± 0.2369
Intercept ± SD	787.9 ± 170.034	1196 ± 105.96
Standard error of slope	0.1375	0.0967
Standard error of intercept	69.43	43.27
95% confidence interval of slope	6.025 – 6.698	6.057 – 6.554
95% confidence interval of intercept	618 – 957.8	1085 – 1308
P value	< 0.0001	< 0.0001

Table II: Accuracy of the proposed method (n=6).

Excess drug added to analyte (%)	Theoretical content (ng)	Conc. found (ng) ± SD	% Recovery	% RSD
Rutin				
0	300	295.17 ± 3.82	98.39	1.29
50	450	446.67 ± 3.93	99.26	0.88
100	600	594.33 ± 4.32	99.06	0.73
150	750	743.17 ± 6.97	99.09	0.94
Aloe emodin				
0	150	294.83 ± 3.97	98.28	1.35
50	300	444.33 ± 4.50	98.74	1.01
100	450	594.50 ± 5.17	99.08	0.87
150	750	744.17 ± 3.71	99.22	0.50

Table III: Precision of the proposed method of Rutin and Aloe emodin

	Rutin					
	Repeatability (Intraday precision)			Intermediate precision (Interday)		
Conc. (ng/spot)	Avg Conc. ± SD (n = 6)	Standard error	% RSD	Avg Conc. ± SD (n = 6)	Standard error	% RSD
300	2649 ± 26	10.63	0.98	2637 ± 24	9.71	0.90
400	3342 ± 34	13.71	1.00	3345 ± 40	16.18	1.18
500	3999 ± 46	18.94	1.16	4018 ± 46	18.93	1.15
			Aloe emodin			
369.0	3057 ± 23	9.34	0.75	3026 ± 40	16.15	1.31
535.5	3057 ± 40	16.27	1.07	3729 ± 46	18.65	1.23
738.0	4441 ± 19	7.74	0.43	4449 ± 27	11.11	0.61

Table IV : Robustness of the proposed HPTLC method of Rutin and Aloe emodin

	Rutin					
Conc. (ng/spot)	Mobile phase composition (ethyl acetate: methanol: water)					
	Original	Used		Area ± SD (n = 3)	% RSD	Rf
400	30:05:04	30:4.9:4.1	-0.1, +0.1	3405 ± 64	1.88	0.35
		30:05:04	0.0	3345 ± 35	1.04	0.33
		30:5.1:3.9	+0.1, -0.1	3349 ± 40	1.18	0.32
			Aloe emodin			
400	30:05:04	30:4.9:4.1	-0.1, +0.1	3723 ± 39	1.04	0.44
		30:05:04	0.0	3724 ± 40	1.08	0.43
		30:5.1:3.9	+0.1, -0.1	3764 ± 37	0.99	0.41

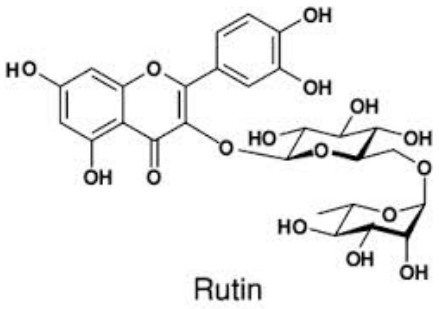

Figure 1. Chemical structure of Rutin

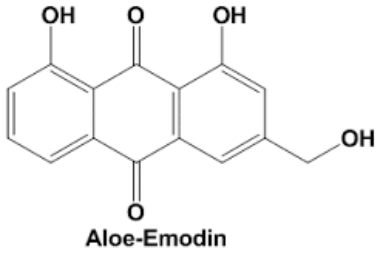

Figure 2. Chemical structure of Aloe emodin

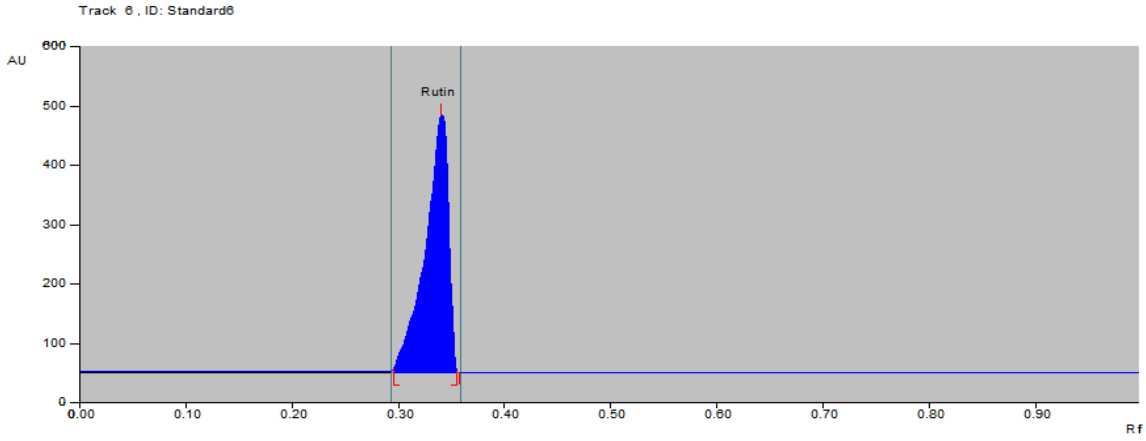

Figure 3. HPTLC chromatogram of standard Rutin

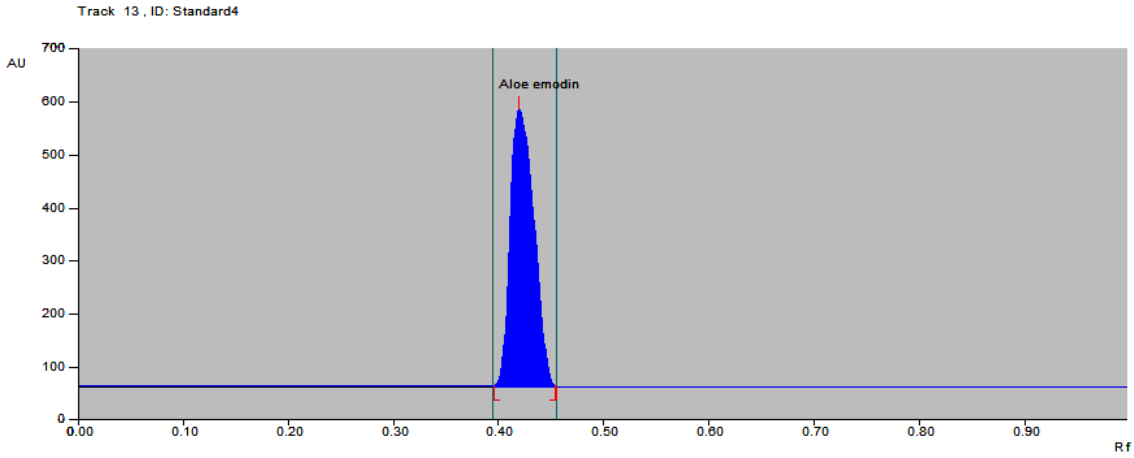

Figure 4. HPTLC chromatogram of standard Aloe emodin

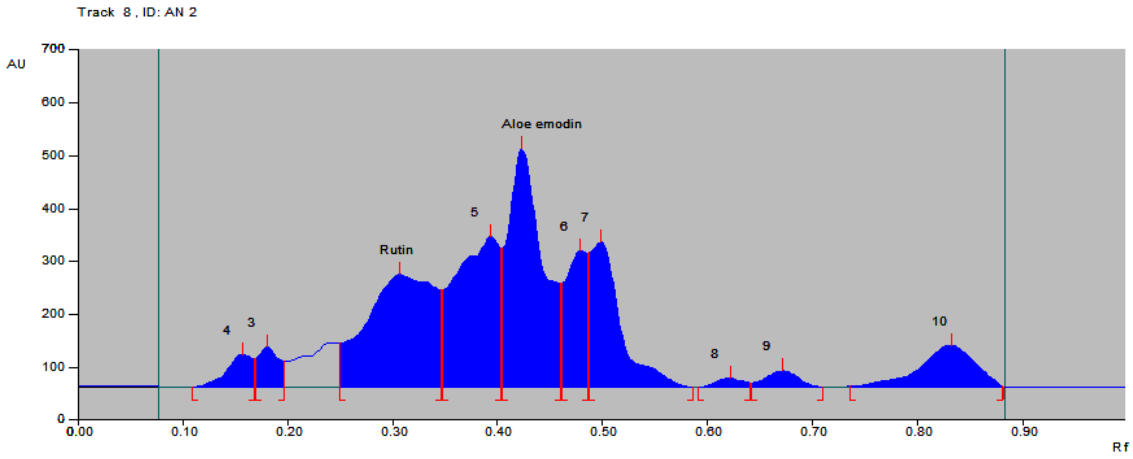

Figure 5. HPTLC Chromatogram Of Methanolic Extract Of Atriplex Nummularia

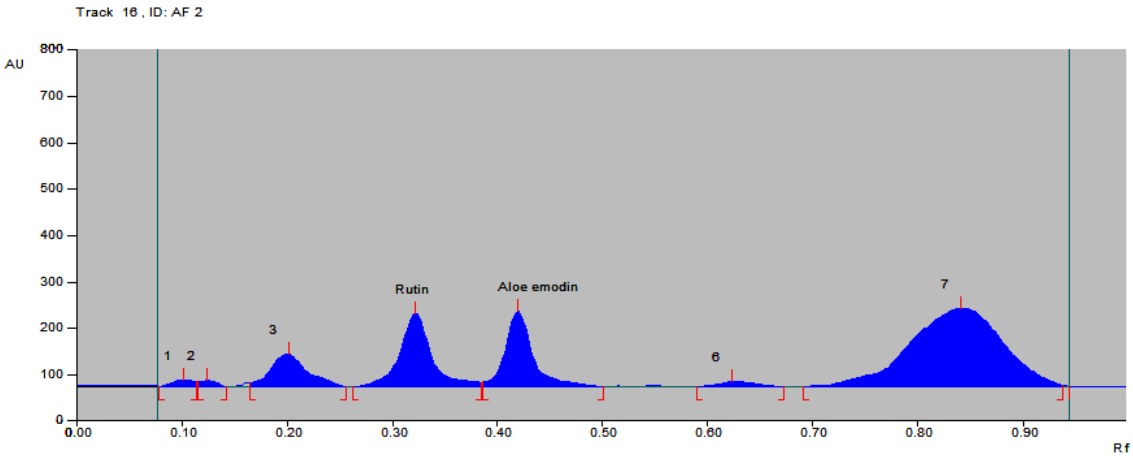

Figure 6. HPTLC chromatogram of methanolic extract of *Atriplex farinosa*

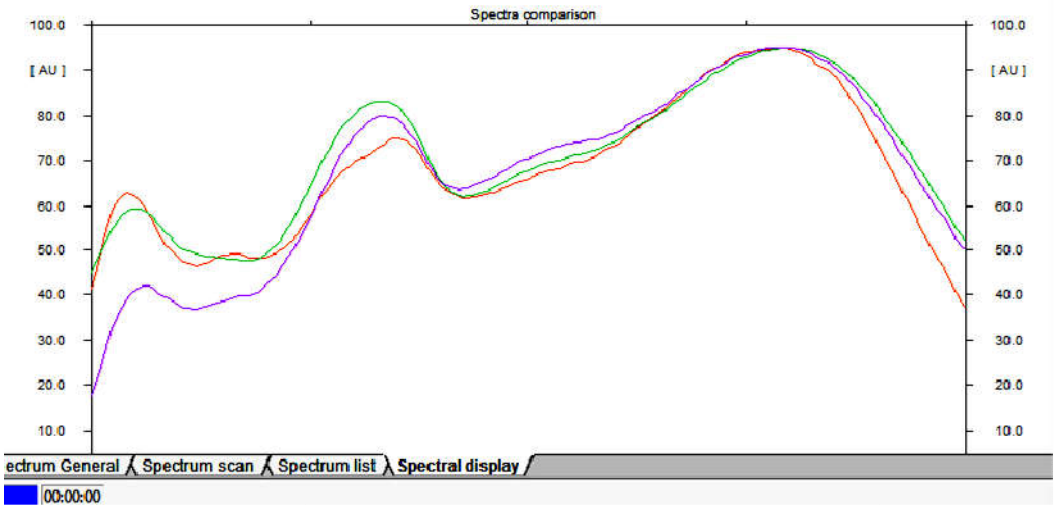

Figure 7. Overlay UV absorption spectra (358 nm) of the standard Rutin andmethanolic extract of *Atriplex nummularia* and *Atriplex nummularia*

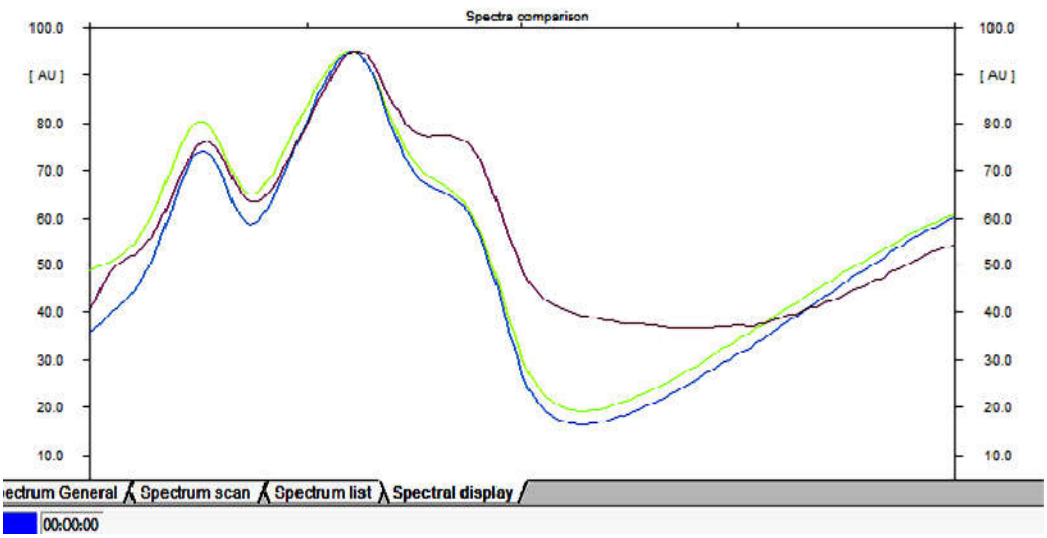

Figure 8. Overlay UV absorption spectra (262 nm) of the standard Aloe emodin andmethanolic extract of *Atriplex nummularia* and *Atriplex farinosa*

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