# Isolation of flavonoids from *Delonix elata* and determination of its rutin content using capillary electrophoresis

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**Abstract**: *Delonix elata* (L.) Gamble (Fabaceae) is an important, traditionally used plant in Saudi Arabia. It is used to relieve rheumatic pain, flatulence and the seeds are employed as purgatives. The aim of the present study was to isolate chemical constituents of the *n*-butanol fraction (BF) of *D. elata* and to find out, by capillary electrophoresis (CE), percentage of rutin present in this BF. Three quercetin glycosides and one kaempferol rutinoside were isolated from the BF of aerial parts of *D. elata*; namely, Quercetin 3-*O*-rutinoside-7-*O*-glucoside (1), Quercetin 3,7-diglucoside (2), Quercetin 3-*O*-rutinoside (RUT) (3) and Kaempferol 3-*O*-rutinoside (4). Rutin, an active constituent has been reported to possess good pharmacological as well as therapeutic potentials. A sensitive and rapid procedure for quantitative determination of RUT by capillary electrophoresis was developed and its content was found to be 7.349 mg/gm, relative to *n*-butanol fraction and 18.373 mg%, relative to the dry powder of *D. elata*. The method could be recommended for approval and use in the pharmaceutical and food industries.

**Keywords**: *Delonix elata*, capillary electrophoresis, flavonoids, rutin.

## INTRODUCTION

*Delonix elata* (L.) Gamble or *Poinciana elata* L. (Fabaceae) is a medium-sized tree that is widely distributed, in India, Oman, Saudi Arabia, Yemen and Egypt, down through Ethiopia, Somalia, Kenya and Tanzania. It is found in Hijaz and Southern regions of Saudi Arabia (Alshanawany, 1996). *D. elata* has been used in the Indian traditional medicine to treat rheumatism, inflammation, jaundice, and bronchial problems (Saravanan *et al*., 2014; Krishnappa *et al*., 2014). Decoction of the leaves and barks is taken to get relief from rheumatic problems like pain and stiffness of the joints, especially affecting the knees (Muruganathan and Mohan, 2011). While in Saudi Arabia, all plant parts are used traditionally to relieve rheumatic pain, flatulence and the seeds are employed as purgatives (Alshanawany, 1996). Previous biological studies on *D. elata* exhibited anti-inflammatory (Manimekalai *et al*., 2011), immune modifying potentials and antioxidant activities (Hegazi, 2011). These activities may be attributed to the presence of gallic acid, ellagic acid, coumaric acid, quercetin, rutin, and Quercetin-3-O-galactoside which were reported in the stem bark and leaves of *Delonix elata* (Sethuraman and Sasikumar, 1995; Krishnappa *et al*., 2014).

Rutin is an important, well known, bioactive phyto constituent. It has strong antioxidant properties, and was proved to lower the risk of heart disease (Jeong *et al*., 2005). Several methods have been developed for the determination of rutin in different plant extracts; these include HPLC (Chen *et al*., 2011; Dresch *et al*., 2014), HPTLC (Abou Donia *et al*., 2006; Amin *et al*., 2013), capillary electrophoresis (Chi *et al*., 2009; Vachirapatama *et al*., 2011), spectrometry (Abou Donia *et al*., 2006), and voltammetry (Magarelli *et al*., 2014). Nowadays, the application of CE for the separation of various active constituents in medicinal plants has become increasingly widespread. Compared with HPLC, CE has advantages of high separation performance, short analysis time, small reagent consumption and easy operation (Chi *et al*., 2009).

To the best of our knowledge, there has been no previous phytochemical work on the Saudi *D. elata*. Moreover, there has been no report on determination of rutin in *D. elata*. In the present study, we report the isolation and identification of four flavonoids from the *n*- butanol fraction (BF) of *D. elata* as well as determination of its biologically active, rutin content using capillary electrophoresis.

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## MATERIALS AND METHODS

***General experimental procedure for isolation of compounds 1-4***

Optical activity was measured on a JASCO P-2000 Series polarimeter (JASCO Corporation, 2967-5, Tokyo, Japan). The$^1$H, $^{13}$C NMR and 2D NMR spectra were recorded on a BrukerAMX-500 spectrometer with tetramethylsilane (TMS) as an internal standard. Chemical shifts are in ppm (δ), relative to tetramethylsilane as an internal standard and scalar coupling constants (J) reported in Hertz. ESI-MS analyses were measured on an Agilent Triple Quadrupole 6410 QQQ LC/MS mass spectrometer with ESI ion source (gas temperature is 350°C, nebulizer pressure is 60 psi and gas flow rate is 12L/min), operating in the negative and positive scan modes of ionization through direct infusion method using $CH_3OH \backslash H_2O$ (1:1 v/v) at a flow rate of 0.4mL/min. Column chromatography was carried out on Sephadex LH-20 (E. Merck, Darmstadt, Germany). Thin layer chromatography (TLC) was performed on precoated TLC plates (Aluminium sheets, RP-18 F254, Merck, Germany); the detection was done at 254 nm and by spraying with 10% ceric sulphate reagent. Solvent systems used were: S1 ($CHCl_3$/MeOH 7:3) on silica gel plates and S2 (50% aqueous MeOH) on silica gel RP-18 plates. HPLC analysis was performed on a Prominence Shimadzu LC Solution, (Kyoto, Japan) and the system equipped with a CBM-20A communication bus module, two LC-10AD pumps, a DGU-20A3 vacuum degasser, a CTO-10A(C) column oven, and an SPD 10A(V) diode array detector.

***Plant material***

The aerial parts of *Delonix elata* (1.0kg) were collected from Eastern region, Saudi Arabia, in 2012. The identity of the plant was verified by Dr. M. Atiqur Rahman, Professor of Taxonomy, College of Pharmacy, King Saud University. A voucher specimen (No. 48) was deposited in the herbarium of Department of Pharmacogonsy, King Saud University.

***Extraction and isolation***

The aerial parts of *D. elata* (1.0kg) were shade-dried, ground and extracted at room temperature with EtOH: $H_2O$ (8:2, thrice). The combined ethanol extract (60g) was divided into *n*-hexane (15g), *n*-BuOH (25g) and water (15g) soluble sub-fractions. A part of *n*-BuOH (BF) soluble fraction (15g) was dissolved in water and loaded on Sephadex LH-20 column and the elution was successively carried out with water and mixtures of $H_2O$ and MeOH in decreasing order of polarity leading to three major sub-fractions I-III. Sub-fraction I, obtained from MeOH-$H_2O$ (2:8) showed one major spot on TLC ($CHCl_3$/MeOH 7:3) along with little impurities and was further purified by HPLC with flow rate 2.0mL/min eluted with MeOH-$H_2O$ (4.0:6.0), to afford compound 1 (10 mg). Sub-fraction II, obtained from MeOH-$H_2O$ (3:7) was a mixture of two compounds which was separated through HPLC with flow rate 1.5mL/min by using MeOH-$H_2O$ (1:1) as eluent, to give compounds 2 (10 mg) and 3 (90mg). The purification of sub-fraction III was carried out on HPLC with flow rate 2.0mL/min using MeOH-$H_2O$ (1:1) as eluent to afford compound (4) (12 mg).

***Acid hydrolysis of Compounds 1-4***

A solution of 5mg in 10ml MeOH-1N HCl (1:1) was boiled under reflux for 4h, concentrated under reduced pressure and diluted with $H_2O$ (10ml). It was extracted with AcOEt and the residue recovered from the organic phase yielded the aglycone. The remaining aqueous layer was neutralized with 5% aq. $NaHCO_3$ solution, concentrated under vacuum and then the sugars were identified by comparative TLC (chloroform/acetic acid/water 3:3.5:0.5) with authentic standards.

***Determination of rutin***

*Instrumentation and electrophoretic conditions*

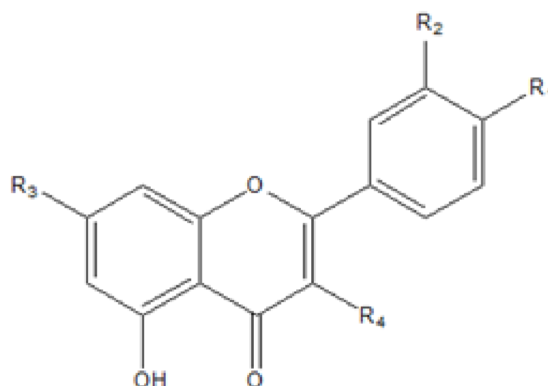
Capillary electrophoresis was performed with an Agilent Capillary Electrophoresis instrument (Agilent Technologies Deutschland, GmbH, Hewlett-Packard-Stress 8, Germany) equipped with temperature controller (Minichiller, Germany) and a diode array detector (DAD). The data handling system comprised of an IBM personal computer and Agilent Chem Station® software. A deactivated fused silica capillary was obtained from Agilent Technology (Fullerton, CA, USA) and had the following dimensions: 65cm total length, 56.5cm effective length and 75µm i.d. The background electrolyte solution (BGE) consisted of borate buffer (40mM, pH 9.2): methanol (90: 10, v/v). The temperature of the capillary and the sample was maintained at 21°C. Detection was performed at 280nm. Samples were injected into the capillary by pressure at the anodic side at 20 mbar for 12 s. The electrophoresis was carried out by applying high voltage (30 kV) to the capillary with the cathode being at the detector end. The capillary was washed between runs with deionized water (3min), then equilibrated with the running buffer (5min) to ensure reproducibility of the assay. The solutions were passed through a Millipore membrane filter (0.2mm) from Nihon Millipore (Japan), before injection.

*Materials and reagents*

Reference standard rutin (Fluka, Sigma Aldrich, GmbH, Germany) was used. Analytical grade solvents and reagents were used all over the work; namely methanol, borax (disodium tetraborate decahydrate), and sodium hydroxide. Deionized water and a Millipore membrane filter (0.2mm) from Nihon, Millipore were used throughout the experiments.

*Standard solutions and construction of calibration graphs*

A stock solution containing 1mg/ml RUT was prepared in methanol. This stock solution was further diluted with




| Compound no. | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| **1** | OH | OH | O-glucoside moiety | O-rutinoside moiety |
| **2** | OH | OH | O-glucoside moiety | O-glucoside moiety |
| **3** | OH | OH | OH | O-rutinoside moiety |
| **4** | OH | H | OH | O-rutinoside moiety |

**Fig. 1**: Chemical structures of compounds 1-4.

borate buffer solution (40mM, pH 9.2) to give working standard solutions within the concentration range (2.5-600 µg/ml). The solutions were stable for at least 2 weeks if kept in a refrigerator. Triplicate injections of each concentration were performed under the optimized CE conditions. The peak area of each standard solution was related to the corresponding concentration to obtain the calibration graph of RUT. Alternatively, the corresponding regression equation was derived.

*Application of the proposed method to the analysis of plant extracts*

Accurately weighted 0.27g of BF was dissolved in 5ml methanol. Volumes of 1.0ml of the methanolic solution were diluted with 1.0ml borate buffer (40mM, pH 9.2) and then injected in triplicates into the CE system at the optimized CE conditions mentioned above. The peak areas recorded for RUT in each sample were used to calculate the amount found in the corresponding BF of *D. elata*.

***Characterization of compounds***

*Quercetin 3-O-rutinoside-7-O-glucoside (1)*

Yellow amorphous powder, complete acid hydrolysis products: quercetin (organic phase); D-glucose and L-rhamnose (aqueous phase). Negative ESI-MS, *m/z* 771.2 $[M-H]^-$. $^1H$ NMR (500 MHz, DMSO-$d_6$): δppm 7.51 (2H, m, H-2`/6`), 6.82 (1H, d, J=9Hz, H-5`), 6.78 (1H, d, J=2.1 Hz, H-8), 6.44 (1H, d, J=2.1 Hz, H-6), 5.33 (1H, d, J=7 Hz, H-1``), 4.39 (1H, brs, H-1```), 5.08 (1H, d, J=7.2 Hz, H-1````), 3.80-3.08 (m, remaining sugar protons), 0.97 (3H, d, J=6Hz, $CH_3$-6```).

*Quercetin 3,7-O-diglucoside (2)*

Yellow amorphous powder, complete acid hydrolysis products: quercetin (organic phase) and D-glucose (aqueous phase). Negative ESI-MS, *m/z* 625.1 $[M-H]^-$. $^1H$ NMR (500 MHz, DMSO-$d_6$): 7.66 (1H, dd, J=8.4, 2.4 Hz, H-6`), 7.54 (1H, d, J=2.4 Hz, H-2`), 6.84 (1H, d, J=8.4 Hz, H-5`), 6.75 (1H, d, J=2.1 Hz, H-8), 6.42 (1H, d, J= 2.1 Hz, H-6), 5.50 (d, J=8.5 Hz, H-1``), 5.16 (d, J=8.5 Hz, H-1```), 3.90-3.30 (m, remaining sugar protons).

*Quercetin 3-O-rutinoside (3)*

Yellow amorphous powder, complete acid hydrolysis products: quercetin (organic phase); D-glucose and L-rhamnose (aqueous phase). Negative ESI-MS, *m/z* 609.1 $[M-H]^-$. $^1H$ NMR (500 MHz, DMSO-$d_6$): δppm 7.53 (2H, m, H-2`/6`), 6.83 (1H, d, J=9Hz, H-5`), 6.38 (1H, d, J=1.5Hz, H-8), 6.19 (1H, d, J=1.5Hz, H-6), 5.33 (1H, d, J=7Hz, H-1``), 4.39 (1H, brs, H-1```), 3.80-3.10 (m, remaining sugar protons), 0.99 (3H, d, J=6Hz, $CH_3$-6```).

*Kaempferol 3-O-rutinoside (4)*

Yellow amorphous powder, complete acid hydrolysis products: kaempferol (organic phase); D-glucose and L-rhamnose (aqueous phase). Negative ESI-MS, *m/z* 593.1 $[M-H]^-$ and 285.3 $[kaempferol-H]^-$. $^1H$ NMR (500 MHz, DMSO-$d_6$): δppm 8.07 (2H, d, J=8.7 Hz, H-2`/6`), 6.87 (2H, d, J=8.7 Hz, H-3`/5`), 6.43 (1H, brs, H-8), 6.21 (1H,

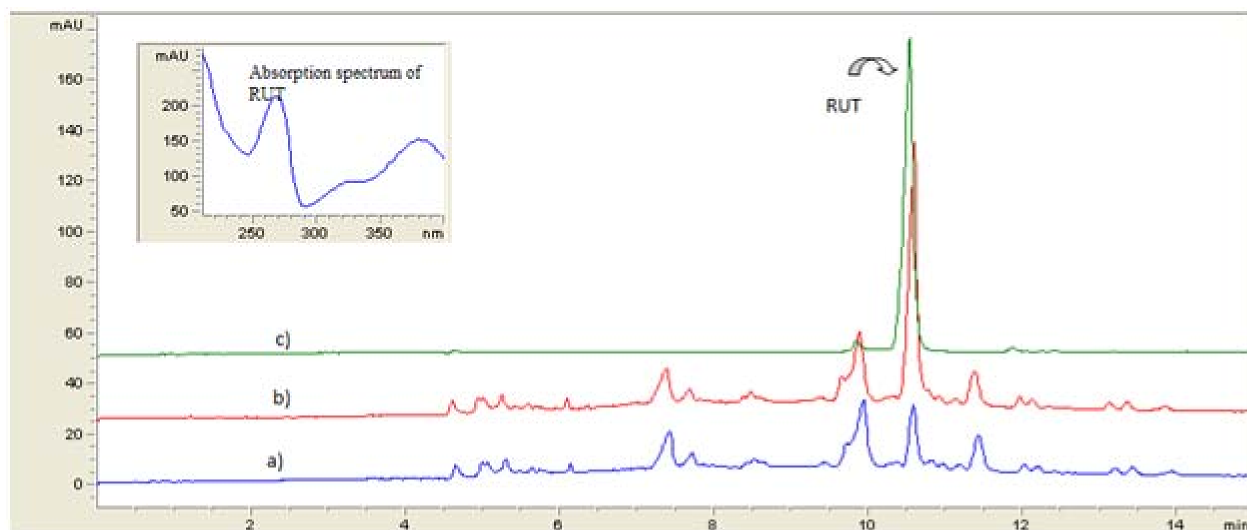



**Fig. 2**: Electropherogram of (a) BF and (b) BF spiked with 400µg/ml of RUT and c) standard RUT solution of concentration 400 µg/ml. The figure also shows the absorption spectrum of RUT.

brs, H-6), 5.36 (1H, d, J=7.8 Hz, H-1``), 4.35 (1H, brs, H-1```), 3.75-3.10 (m, remaining sugar protons), 1.10 (3H, d, J=6Hz, $CH_3$-6```).

## RESULTS

Three quercetin glycosides and one kaempferol rutinoside were isolated from the BF of aerial parts of *D. elata* after fractionation on a Sephadex LH-20 column, followed by several HPLC separations namely, Quercetin 3-*O*-rutinoside-7-*O*-glucoside (1), Quercetin 3,7-diglucoside (2), Quercetin-3-*O*-rutinoside (3) and Kaempferol 3-*O*-rutinoside (4) (fig. 1). All isolated compounds are reported for the first time from Saudi *D. elata.* Rutin (3), being an important, bioactive constituent and a major compound in *D. elata*, it was of interest to determine its content in the plant using CE. The content of RUT was found to be 7.349mg/gm, relative to *n*-butanol fraction and 18.373mg%, relative to the dry plant material.

## DISCUSSION

Based on the chromatographic properties of compounds 1-3 (Mabry *et al*., 1989), they were expected to possess quercetin-like structure. Two characteristic spin coupling systems were distinguished in their $^1$H NMR spectra. The first system was recorded as a *meta* doublet at 7.51-7.66, a doublet of doublet at 7.53-7.54 and an *ortho* doublet of one proton at 6.82-6.84, which were assignable to H-2`, 6` and 5` of 3`, 4`-dihydroxy B-ring. The second coupling system was described as two *meta*-coupled doublets at 6.38 and 6.19 for H-8 and H-6 of 5,7-dihydroxy A-ring, respectively (Mabry *et al*., 1989; Agrawal and Bansal, 1989; Williams and Harborne, 1994).

On the basis of the chromatographic properties, compound 4 was expected to be kaempferol-like structure. $^1$H NMR spectrum showed in its aromatic region two spin coupling systems, each of two doublets. The first one consists of two *ortho* doublets at 8.07 and 6.87 assigned to H-2`/6` and H-3`/5`, respectively of 4-hydroxy B-ring. Whereas the second system appeared as two *meta*-coupled protons, which were characterised to H-8 and H-6 of 5, 7-dihydroxy A-ring at δ 6.43 and 6.21, respectively (Williams and Harborne, 1994). The sugar moieties were identified based on their characteristic resonances and by comparison with the reported data in the literature. Thus, the accurate structures were elucidated by comparative thin layer chromatography (CoTLC) with authentic samples, chemical degradation, and comparison of their spectroscopic data (ESI-MS, NMR) with literature (Materska, 2008; Iwashina *et al*., 2012).

***Optimization of electrophoretic conditions***

Due to the rather complex character of plant samples and the inherent structural similarity of most flavonoids, the method should selectively determine the particular compound without any interference. Thus in an attempt to separate RUT peak from the complex *n*-butanol fraction, practical trials concerning variation of all parameters that affect the electrophoretic separations were carried out. These included composition of BGE, applied voltage and pressure, injection time, cartridge temperature, and even the inclusion of surfactants in BGE. Preliminary trials were based on the previously reported paper dealing with the determination of RUT in Buckwheat Tea and *Fagopyrum tataricum* seeds by CE (Vachirapatama *et al*., 2011). In this article, RUT was successfully determined using borate buffer at pH 9.4 as the BGE. Thus, BGE consisting of borate buffer (20mM, pH 9.4) was initially

tested for its ability to separate RUT peaks from the complex sample matrix of *D. elata* BF. However, this BGE could not provide complete baseline separation of RUT peak from adjacent peaks. Thus borate buffer solutions of varying pH (9.4-11.5) were tried as BGE where pH 10.0 provided higher separation efficiency compared to pH 9.4. Moreover, the effect of borate concentration on the separation efficiency was tested using borate buffer solutions of varying concentrations (5-50mM) at a constant pH of 10.0. It was found that borate buffer strength of 40mM produced the sharpest RUT peaks but still with poor peak purity. Thus, inclusion of methanol (5-20%) as an organic modifier was tested in an attempt to improve the resolution of RUT from adjacent peaks (Alzoman *et al*., 2013). It was noticed that 10% methanol in the BGE provided complete baseline separation and highly pure RUT peaks. Also, an increase in the applied voltage from 15 to 40 kV was tried where 30KV produced the best compromise between resolution, peak shape, and run time.

**Table 1**: Regression and statistical parameters for the determination of RUT by the proposed CE method.

| Parameter | RUT |
|---|---|
| Linearity range (μg / mL) | 2.5-600 |
| LOQ[a] (μg / mL) | 1.50 |
| LOD[b] (μg / mL) | 0.60 |
| Intercept | 0.2756 |
| Slope | 1.6496 |
| Correlation coefficient | 0.9998 |
| $S_a$[c] | 1.3098 |
| $S_b$[d] | 0.0080 |
| $S_{y/x}$[e] | 2.9448 |

[a]LOQ: Limit of quantitation, [b]LOD: Limit of detection. [c]$S_a$: Standard deviation of intercept, [d]$S_b$: Standard deviation of slope, [e]$S_{y/x}$: Standard deviation of residuals.

In addition, a variation in the applied pressure (40-60 mbar) and in the injection time (5-30s) was tested and best results were obtained using a pressure of 20mbar/12 s (time of injection). Since capillary temperature control is extremely important for the reproducibility of the assay (Chi *et al*., 2009; Magarelli *et al*., 2014), a convenient operational temperature of 21°C was selected as the optimal temperature regarding peak shape, run time, and most importantly the resolution from the background interfering peaks that were found in the analyzed plant extracts.

In conclusion, the best separation of RUT in BF was achieved using the following optimized conditions: A fused silica capillary of 56.5cm effective length, 75μm ID, BGE consisting of borate buffer (40mM, pH 10.0) and methanol (90:10, v/v), 21°C working temperature, 30 KV, and 20 mbar pressure/ 12 sec injection time. An electropherogram of standard RUT with migration time of 10.53±0.32 min under optimal condition was shown in fig. 2c.

**Table 2**: Intra-day and inter-day precision for the determination of RUT by the proposed CE method

| | Intra-day precision[a] | Inter-day precision[b] |
|---|---|---|
| Concentration (μg/ml) | RSD | |
| 400 | 0.35 | 0.98 |
| 200 | 1.69 | 1.88 |
| 5 | 1.62 | 1.91 |

[a]Data are based on assay of three determinations in the same day, [b]Data are based on assay of three replicates on three different days.

**Table 3**: Recovery results for the determination of RUT by the proposed CE method

| Concentration spiked (μg/ml) | Mean recovery (%) ± RSD[a] | $E_r$ (%)[b] |
|---|---|---|
| 250 | 98.75±1.22 | 1.25 |
| 125 | 97.88±0.95 | 2.12 |
| 50 | 100.09±1.55 | -0.09 |

[a]RSD of three determinations, [b]Percentage relative error.

***Method validation***

The method was validated according to the ICH guidelines (1996; 2002). Linearity of the method was evaluated by relating the peak area of standard RUT to its concentration within the concentration range stated in table 1. Using the method of least squares, regression equations, correlation coefficients (r), intercepts (a), slopes (b), standard deviation of residuals ($S_{y/x}$), standard deviation of intercepts ($S_a$), and standard deviation of slopes ($S_b$) were calculated. The high values of the correlation coefficients with negligible intercepts indicate the good linearity of the calibration graphs (Miller, 2000).

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated on the basis that they provided S/N ratio of 3 or 10 with a RSD value less than 10%, respectively (Miller, 2000). The calculated values for RUT were listed in table 1.

Precision was evaluated by analyzing three different concentration levels of RUT (5.0, 200.0, and 400.0μg/ml) either on the same day for intraday precision (method repeatability) or on three different days for interday precision (intermediate precision). Low RSD values, less than 2%, obtained for both cases indicate a high degree of precision of the proposed method (table 2).

In order to verify the applicability of the proposed method for the analysis of RUT in prepared extracts of *D. elata*, recovery testing of spiked samples was carried out. Constant volumes of prepared plant extract were

separately spiked with different known concentrations of standard RUT. These solutions were then analyzed under the optimized conditions and the peak areas obtained for RUT were recorded and related to RUT concentration. The results of the recovery assays indicated that the method was accurate enough for the analysis of RUT in *D. elata n*-butanol fraction (table 3). Comparing the peak area of RUT added to plant extract with that of a standard solution at the same concentration levels, low, medium, and high indicated that the matrix had nearly no effect on actual analysis.

Method robustness was evaluated by carrying out the analysis under small and deliberate changes of experimental parameters including the pH of borate buffer (9.8, 10.0, 10.2), percentage of methanol in the BGE (8, 10, 12%), applied voltage (28, 30, 32 KV), and capillary temperature (20, 21, 23$^{o}$C). The low values of RSD ($<$ 2%) of peak areas, along with nearly unchanged migration times obtained for RUT following these changes indicated the robustness of the method.

Specificity of the method for the analysis of RUT in BF was assessed by spiking the prepared plant extract with working standards of RUT along with calculating the peak purity, a property of DAD. High values of recoveries, along with high degrees of peak purity indicated the ability of the method to determine RUT selectively in BF without any potential interference from other components in the plant extract. The peaks obtained for RUT had clear baseline separation and were obtained within reasonable migration times. Representative electropherograms of prepared *Delonix n*-butanol fraction before and after spiking with standard RUT are shown in figs. 2a,b.

***Application of the CE method for the analysis of plant material***

The optimized CE method was applied to quantitative assay of RUT in BF. The method allowed complete base-line separation of RUT peaks without any interference from existing peaks in the BF (figs. 2a-c). The content of RUT was found to be 7.349mg/gm, relative to *n*-butanol fraction and 18.373mg%, relative to the dry plant material.

## CONCLUSIONS

Rutin is important in the body to strengthen arteries, veins (Danila *et al*., 2007) and to harden bones and teeth. It is found in many plants, fruits and vegetables (Wu *et al*., 2007). Moreover, rutin can inhibit platelet aggregation thus making the blood thinner which improves the circulation. It also inhibits aldose reductase activity, leading to delay of diabetes (Alsaif, 2009). Thus, the traditional uses and the proved biological activities of *D. elata* could be attributed to its high rutin content. The CE method developed for the quantitative determination of RUT in *D. elata* was rapid, selective and highly sensitive, which recommends its use in quality control procedures for the determination of RUT in different plant samples without interference.

## ACKNOWLEDGEMENTS

The authors would like to extend their appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the research group project No. RGP-VPP-331.

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