Wound healing effect and flavonoid contents of three selected *Byrsonima* species

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Abstract

The present study aimed to develop a methodology for quantification of prepared samples by analysis using capillary electrophoresis to analyze apigenin, apigalacatequin and kaempferol from methanol extract fruits and seeds of three selected *Byrsonima* species. The effect of methanolic extracts from these Malpighiaceae species was assayed in streptozotocin-induced diabetic rats. Methanol extract in a topical form accelerates the wound healing process by decreasing the surface area of the wound with a significant increase in the rate of wound contraction and tensile strength. *B. crassifolia* was most effective of the three species tested in promoting diabetic wound healing in rats through the processes of tissue regeneration.
Keywords: Byrsonima crassifolia, B. bucidaefolia, B. bahiana, capillary electrophoresis, wound healing, flavonoids.

1 Introduction

The *Byrsonima species* is a tropical tree, distributed widely in México, Central and South America. The fruit is edible and bright yellow when ripened; it has sweet taste and slightly bitter aftertaste. In México, the fruit is consumed as juice, liquor, jelly and candy. Since prehispanic times it has been used as medicine in treating various diseases [1]. Phytochemical studies indicate that these plants contain esters [2], epicatechins [3], glycolipids [4]. Studies aiming to describe the diverse biological activities of the *Byrsonima* species have shown promising results as spasmogenic [5], anti-inflammatory [6], antioxidant [7, 8], antibacterial [9], trypanocidal [10] and hypoglycemic [11]. Based on traditional

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uses of seeds from *B. crassifolia* the objective of this work was to establish the chemical composition and wound healing activity of methanol extracts fruits and seeds of *B. crassifolia, B. bucidaefolia* and *B. bahiana*.

2 Materials and methods

2.1 Reagents and standards solutions

Flavonoid standards, apigalacatequin, apigenin, and kaempferol were purchased from Sigma Chemical Co. (St Louis, MO, USA).

2.2 Plant material

Byrsonima crassifolia L, *B. bucidaefolia* and *B. bahiana* belong to the Malpighiaceae family, fruits were collected in Tlacomulco (México State), Alarcon (Guerrero State) and Cuautla (Morelos State) respectively in the month of July 2011 and were taxonomically authenticated in the Herbario of Escuela Nacional de Ciencias Biologicas, Instituto Politécnico Nacional. A voucher specimen of these plants is stored for reference.

2.3 Preparation of extracts

Air-dried and powdered fruit and seeds from *B. crassifolia* (400 g/batch) separately were extracted twice for 2 h with 1.5 l methanol in a Soxhlet apparatus. The extracts after filtered were concentrated under vacuum at 30°C in a rotary evaporator (Buchi XYZ Flawil, Switzerland).

2.4 Determination of phenolic content (PT)

The total phenolic content in the methanol extracts was measured using Folin-Ciocalteu reagent method [12].

2.5 Capillary electrophoresis analyses

The determination of flavonoids were carried out on a Beckman PACE/MDQ capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA), equipped with an on-column diode-array detection (DAD) system set at 214 nm and a temperature control device set at 25ºC. Software for data acquisition and treatment (32 Karat™ Software v 7.0) was used for peak integration and data analysis. Samples were introduced onto the capillary via hydrodynamic injection by applying 0.5 psi for 10 s. A constant voltage of 15 kV was used for all experiments. Uncoated fused-silica capillaries (CAP Capillary Tubing Beckman Coulter) with an inner diameter of 75µm and a total length of 60 cm were used.

New capillaries were conditioned with a 1 mol NaOH solution (10 min), followed by deionized water for 5 min, and finally the BGE for 30 min. Between runs, the capillary was rinsed for 2 min with 1mol, NaOH, followed by water for 2 min and equilibrated with running buffer for 5 min and buffer. The extract was

dissolved in methanol/water (1:1 v/v) and filtered (pore size, 0.45 μm; Millipore). The running buffer used was 40mM borate and 40mM sodium dodecyl sulfate, pH 9.0.The identification of apigenin, apigalacatequin and kaempferol in the samples was performed by comparing their retention times with those of authentic standard and by addition of standard solutions to the sample analyzed by capillary electrophoresis and by comparison of their UV-Vis spectrum.

2.6 Determination of the detection and quantification limits

The detection limits were determined by injecting (n = 5) solutions of apigenin, apigalacatequin and kaempferol of known concentration (10 μl each) and then decreasing the concentrations of the samples until a peak is detected having a signal/noise ratio of 3. The corresponding concentration was considered as being the minimal detectable concentration. The quantification limits were determined by performing the same methodology and thus, the quantification limit was considered to be the chromatographic peak having a signal/noise ratio of 10.

2.7 Analytical curves

The content estimation of the apigenin, apigalacatequin and kaempferolin in the samples was performed by external calibration. The compounds in the study were dissolved separately in spectroscopy grade methanol in order to obtain stock solutions, which were appropriately diluted for each of the substances. Aliquots of 10 μl dilutions for each standard were analyzed via capillary electrophoresis with each determination being carried out five times. For each standard, the corresponding chromatogram was obtained and a graphical plot was constructed of the mean of areas of the chromatogram against the concentration for each substance. A linear least-square regression of the peak areas as a function of the concentrations was performed to determine the correlation coefficients. The equation parameters (slope and intercept) of each standard curve were used to obtain the concentration values for the samples. Specimens with an analytic concentration exceeding the analytical curve were reassayed upon appropriate dilution of the samples.

2.8 Linearity

The analytical procedure was verified from the linearity of the assayed method in terms of the correlation coefficient obtained, which was evaluated by analyzing each sample with known amount of the analyte at low, medium and high concentrations. The aliquots (10 μl) were analyzed via capillary electrophoresis as described above. Each determination was carried out five times. For each spiked sample, the corresponding chromatograms were obtained and a plot of the average areas against their concentrations was constructed. Linear least-square regression was performed to determine the correlation coefficients.

2.9 Accuracy and precision

The accuracy of the assayed method was evaluated by performing replicate analyses against an analytical calibration curve and calculating the mean percentage differences between the theoretical values and the measured values.

The accuracy values in the inter-day variation studies using capillary electrophoresis at low, medium and high concentrations of apigenin, apigalacatequin and kaempferol were evaluated in the extracts. The precision of a method is expressed as the percentage of the coefficient of variation (CV) of the replicate measurements. The precision of the method was tested for inter-day repeatability via capillary electrophoresis. The inter-day variability of the method was determined from three different analysis (n = 5) for each sample with an addition of known amounts of analyte at low, medium and high concentrations.

2.10 Experimental animals

This study was conducted in Wistar rats with 150 to 200 g weight. All were procured from bioterio of ENCB-IPN and were housed in microlon boxes in a controlled environment (temperature 25 ± 2°C) with standard laboratory diet and water *ad libitum*. Animals were acclimatized for three days in that new environment prior to the experiments. Litter was discarded from cages and the floor renewed thrice a week to ensure hygiene and comfort for animals. The ethical clearance in animal handling was observed as described in NIH publication No. 85-23 revised 1985.

2.11 Wound healing activity

Severe diabetes mellitus was induced in overnight fasted male rats by a single intraperitoneal injection of streptozotocin, at a dose of 50 mg/kg body weight dissolved in cold citrate buffer (pH 4.5) [13]. Rats with permanent high fasting blood glucose level > 300 mg/dl were included for the experiments. In the experiment a total 54 diabetic rats were divided into nine groups (n = 6 per group): Group I: normal control treated topically with simple ointment, Group II: diabetic control treated topically with simple ointment, Group III to VIII: diabetic treated topically with methanol extract of B. crassifolia fruit (NFC) and seeds (NSC), NFG (fruit) and NSG (seeds) for B. bucidaefolia, and B. bahiana NFT (fruit) and NST (seeds). Group IX: diabetic treated topically with nitrofurazone (0.2% w/w).

The back of each diabetic rat was shaved under Nembutal (35 mg/kg, ip) anesthesia and open circular wounds were produced on each rat by excising the skin. The wound area was measured immediately by placing transparent tracing paper over the wound and tracing it out [14]. After the wound creation, the rats were subdivided into nine groups: Group I: normal control treated with simple ointment, Group II: diabetic control treated with simple ointment, Group III: diabetic treated topically with nitrofurazone (0.2% w/w). Group IV–VIII: diabetic treated topically with methanol extract of fruits and seeds (10% w/w).

The rate of wound contraction was calculated by the use of a planimeter on respective days (3, 6, 9, 12, 14) after wound creation and were expressed as a percentage of the initial wound size. The number of days required for healing of the scar without any residual of the raw wound determined the period of epithelialization.

2.12 Statistical analysis

All values are expressed as means ± S.D. and statistical analyses were performed by means of the Student's or by one-way ANOVA, and then differences among means were analyzed with Fisher's Protected Least Significant Difference multiple-comparison test. Differences at $p<0.05$ were considered to be significant.

3 Results

Fruit diameter ranged from 0.7 cm to 1.5 cm, total polyphenols from 0.037 to 0.048 mg gallic acid equivalents (GAE)/g fruit and 0.046 to 0.068 mg gallic acid equivalents (GAE)/g seeds. In this study, three major flavonoids were detected in the methanolic extract of the fruits and seeds from *Byrsonima* different species.

A number of preliminary capillary electrophoresis experiments employing samples were performed to establish optimal conditions for the analysis of apigenin, apigalacatequin and kaempferol (Fig. 1). Calibration curves for flavonoids ranging from 0.10 to 50.0 $\mu g/ml^{-1}$ were determined by linear regression. In some cases, resorcinol was added in the separation as an internal standard to ensure the system is in proper conditions during these analyses. The results of the regression equations of calibration curves and detection limits for the three flavonoids are summarized in Table l.

The linearity of the method was determined by linear regression.

Table 1: Regression data of the analytical calibration curves for determination of apigenin, apigalacatequin and kaemferol via capillary electrophoresis.

	Parameter values		
	Apigenin	Apigalacatequin	Kaemferol
LR (μg/ml)	0.10-50	0.10-50	0.10-50
a	0.0638	0.0121	0.0567
b	0.0136	0.0115	0.0119
Sa	0.0034	0.0023	0.0024
Sb	0.0012	0.0015	0.0011
r	0.9996	0.9996	0.9996
n	10	10	10

LR: linear range, *b:* slope, *a:* intercept, *Sb:* standard deviation of the slope, *Sa:* standard deviation of the intercept, *r:* correlation coefficient, *n:* number of samples. Linear regression, formula: y = a + bx, where y = ratio of peak areas, x = concentration (μg/ml), *a* = intercept and *b* = slope.

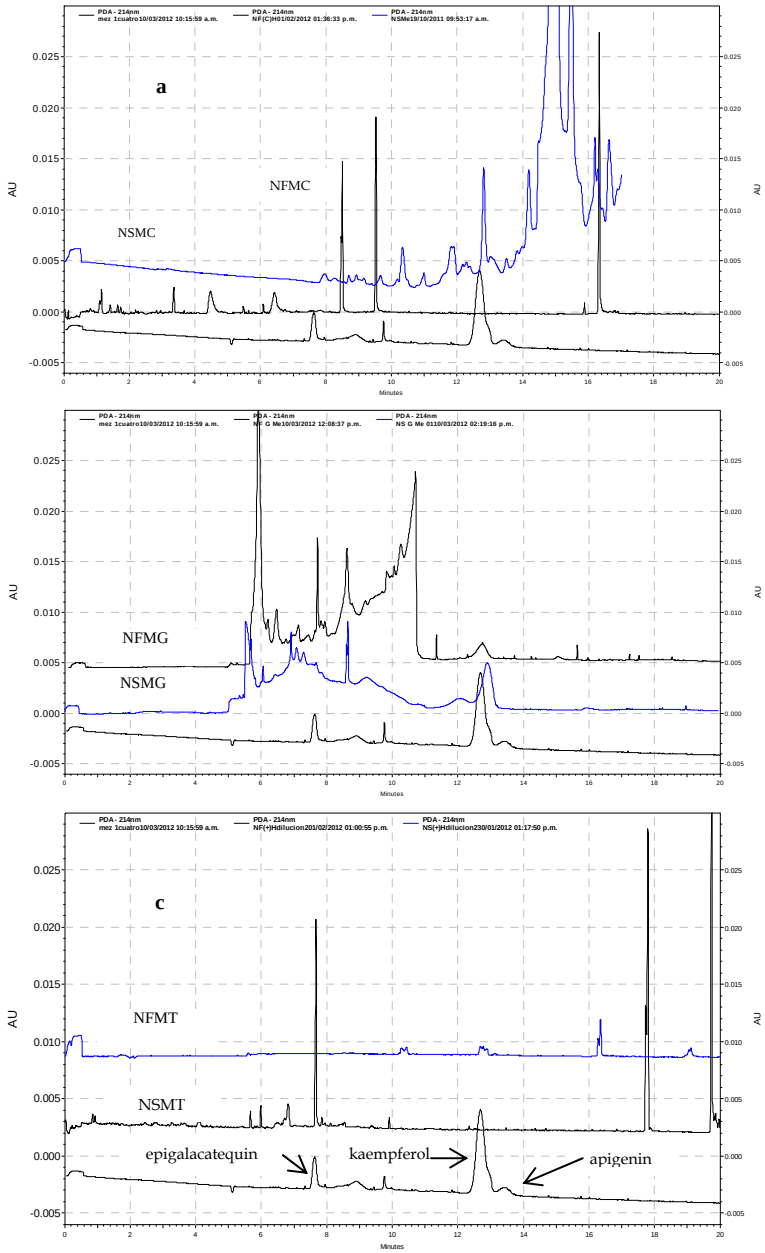

Figure 1: Analytical capillary electrophoresis of flavonoids recorded at 214 nm. Epigalacatequin (Tr=7.16 ± 0.15 min); kaempferol (Tr= 12.32 ± 0.64 min); apigenin (Tr=11.37 ± 0.52min). For the conditions, see the experimental section.

The analysis of the samples spiked with known amounts of analyte demonstrated that the response was proportional to the concentrations of the samples with the determination coefficient being r^2= 0.9996 for the linear range of the analytical calibration curves for the samples.

The precision of method is expressed as the percentage of the coefficient of variation (CV) of the replicate measurements. The accuracy values were less than 5% (Table 2). Regarding the precision of the assay, the interday coefficients of variation were less than ± 5%. In this work, the precision of the method was tested for the inter-day repeatability of the samples. The inter-day variability of the assayed method was determined at low, medium and high concentrations. The results are shown in Table 2. These data indicate that the method is reproducible. This method does not require tedious procedures to eliminate interfering materials. Validation experiments showed good precision, to a p value of 0.05.

Table 3 reports the results of total phenolic contents (TP) analyses. TP contents assay of fruits showed that the methanol extract had lowest phenolic

Table 2: Interday accuracy and precision of the capillary electrophoresis for the determination of apigenin, apigalacatequin and kaemferol.

Apigenin (µg/ml)				Apigalacatequin (µg/ml)			Kaemferol (µg/ml)		
Conc added	Conc Found	AC (%)	CV (%)	Conc Found	AC (%)	CV (%)	Conc Found	AC (%)	CV (%)
1	0.99 ±0.06	1.10	2.19	1.01 ± 0.04	2.00	3.92	1.02 ± 0.04	3.00	3.67
25	24.61 ± 1.75	3.40	4.65	24.52 ± 1.75	1.92	2.78	24.36 ± 1.75	0.56	2.98
50	49.41 ± 2.53	1.18	2.51	49.38 ± 2.53	0.24	2.23	49.37 ± 2.53	1.26	2.74

Conc: concentration (µg/ml), CV: coefficient of variation, Ac: accuracy.

Table 3: Concentrations of total phenolics (TP)

Sampling	TP (mgGAE/g extract)
NFC	0 481 ± 0.66
NSC	0.516 ± 0.033
NFT	0.430 ± 0.012
NST	0.683 ± 0.007
NFG	0.372 ± 0.091
NSG	0.468 ± 0.025

Results are means ± SD.

contents of 0.481±0.66 to 0.372±0.091 mg GAE/g extract sapper low if compared with other fruits. Seeds presented low TP contents of 0. 683±0.007 to 0.468±0.0 25mg GAE/g extracts. Indeed, the profile of the phenolic compounds does not show significant differences when comparing fruits and seeds of *Byrsonima* of three different species.

The results of the present study revealed that the application of extract of B. crassifolia fruit (NFC) and seeds (NSC), NFG (fruit) and NSG (seeds) for B. bucidaefolia, and B. bahiana NFT (fruit) and NST (seeds) on the intentionally excised wound surface at doses of 10% w/w on streptozotocin-induced diabetic rats accelerated the wound healing process by decreasing the wound area. The wound contractions at different days are shown in Table 4.

It was also observed that epithelialization period of treated and standard group were better in comparison to simple ointment base treated group (Fig. 2). However, methanol extracts of *B. crassifolia* and *B. bahiana* reduced epithelialization period on day 14 ($p<0.05$), nitrofurazone reduced epithelialization period on day 18 (data not shown) ($p< 0.05$) in comparison with the control group on day 22 (data not shown). It has been observed that the rate of healing of wounds in diabetic patients is slow compared to healthy patients. Due to the unknown molecular mechanism involved and lack of successful evidence, treatment of diabetic wounds remains one of the greatest challenges for the clinician, making it important to understand the mechanism involved in the impaired skin wound healing in diabetes. The results obtained from the present study showed positive signs in potentiating rate of wound healing in diabetic rats when compared with untreated rats (Fig 2). Methanol extract increase rate of wound contraction, rate of epithelialization period. The rate of wound contraction was found to be significant in topically treated rats. Furthermore, rate of contraction determines the period of epithelialization, which can be defined as the centripetal movement of the edges of a full-thickness wound to facilitate closure of the defect [15].

4 Discussion

In conclusion, the capillary electrophoresis analysis of the samples was developed for the simultaneous determination of apigenin, apigalacatequin and kaempferol fruits and seeds of *Byrsonima* of three different spices providing a method for their analysis. This method does not require tedious procedures to eliminate interfering materials. Validation experiments showed good precision and accuracy for the method with the coefficients of variation being less than ± 5%. The wound healing activity results presented here demonstrate that this methanol extracts from fruits and seeds of *Byrsonima crassifolia, B. bucidaefolia* and *B. bahiana* showed difference in the concentrations of the main bioactive compounds in fruits and seeds, can be opportunely defined on the basis of chemical, agricultural and environmental knowledge.

Table 4: Effect of topical application of hexane extract of fruits and seeds of *Byrsonima crassifolia*, *B. bucidaefolia* and *B. bahiana* on the wound area (mm^2) over a period of 14 days in rats.

	Wound closure mm^2								
Days	Control normal	Control Diabetic	NT	NFT	NST	NFG	NSG	NFC	NSC
3	130.6 ± 2.14 (48%)	59.7 ± 7.18 (22%)	165.4 ± 5.15 (51%)	130.6 ± 4.87 (48%)	130.0 ± 1.18 (48%)	120.8 ± 4.85 (45%)	140.8 ± 4.25 (52%)	150.2 ± 7.21 (55%)	147.8 ± 2.54 (55%)
5	138.6 ± 4.66 (51%)	75.1 ± 3.46 (28%)	168.2 ± 1.45 (63%)	160.0 ± 5.41 (59%)	144.5 ± 4.58 (53%)	135.2 ± 5.01 (50%)	153.2 ± 5.85 (57%)	193.6 ± 5.46 (72%)	166.2 ± 7.54 (61.5%)
7	144.4 ± 3.70 (53%)	115.8 ± 2.87 (43%)	204.0 ± 4.65 (75%)	220.5 ± 2.14 (59%)	218.1 ± 7.58 (81%)	259.5 ± 7.12 (96%)	242.7 ± 3.06 (90%)	234.1 ± 3.04 (87%)	190.5 ± 2.44 (70.5%)
9	158.0 ± 3.25 (55%)	156.5 ± 5.64 (58%)	215.5 ± 4.96 (80%)	242.7 ± 6.41 (91%)	222.4 ± 4.96 (82%)	266.5 ± 5.07 (99%)	264.5 ± 7.41 (98%)	258.2 ± 1.07 (95%)	220.5 ± 6.35 (82%)
12	182.0 ± 5.41 (67%)	190.3 ± 4.57 (70%)	248.2 ± 3.16 (90%)	270.0 ± 1.28 (100%)	260.7 ± 3.25 (96%)	270.0 ± 4.15 (100%)	270.0 ± 3.07 (100%)	262.6 ± 5.06 (97%)	260.0 ± 2.44 (96%)
14	242.4 ± 8.63 (90%)	234.2 ± 4.12 (86%)	270.0 ± 5.22 (100%)	270.0 ± 5.22 (100)	270.0 ± 5.22 (100%)	–	–	270.0 ± 6.08 (100%)	270 ± 2.41 (100%)

Calculated on the original wound size of 270 mm^2; Value are mean ± S.D. (n = 6 animals in each group); Statistically significant $p<0.05$ compared to group control (ANOVA) followed by Dunnett's test. () percentage wound closure is calculated with respect to respective day 0 wound area. Nitrofurazone (NT) as positive control.

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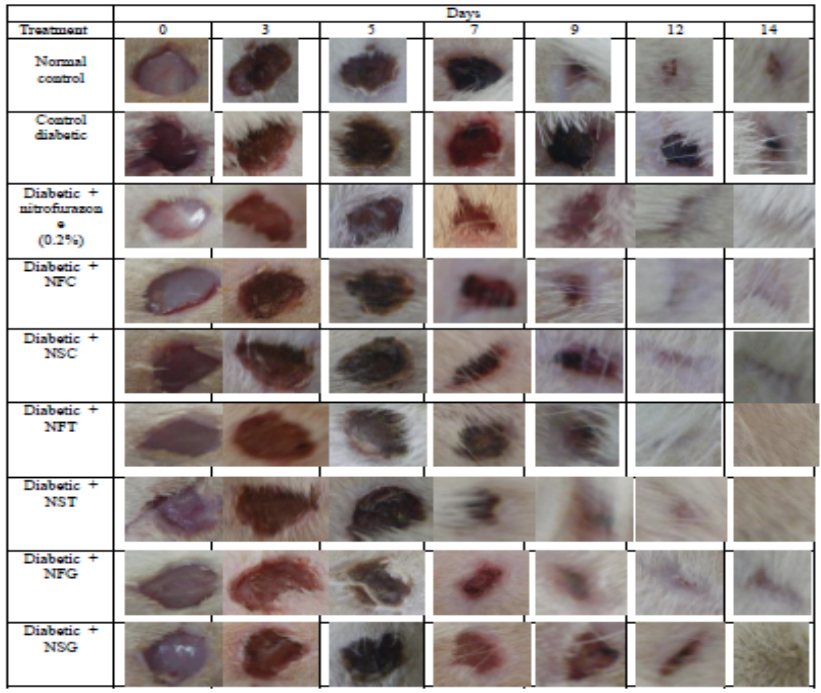

Figure 2: Images of the effect of methanol fruits and seeds extract of *B. crassifolia*, *B. bucidaefolia* and *B. bahiana*, on wound healing in rats on topical treatment.

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