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ORIGINAL ARTICLE

Development and Validation of a Simultaneous RP-HPLC-UV/DAD Method for Determination of Polyphenols in Gels Containing *S. terebinthifolius* Raddi (Anacardiaceae)

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ABSTRACT

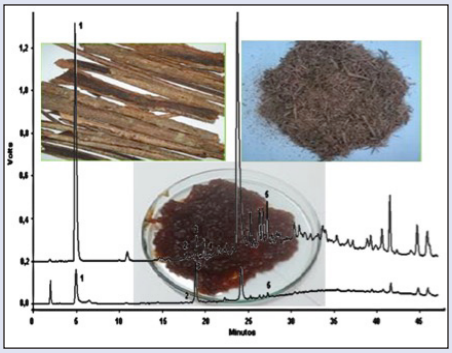
Topical gels containing extracts of *Schinus terebinthifolius* have been used to treat bacterial vaginosis. It has been reported that this species has anti-microbial, anti-inflammatory and anti-ulcerogenic properties, which can be attributed to the presence of phenolic compounds. In this work, a sensitive and selective reversed-phase HPLC-UV/DAD method for the simultaneous assay of six polyphenols that could be present in *S. terebinthifolius* was developed. The method was shown to be accurate and precise. Peak purity and similarity index both exceeded 0.99. Calibration curves were linear over the concentration range studied, with correlation coefficients between 0.9931 and 0.9974. This method was used to determine the polyphenol content of a hydroalcoholic extract and pharmacy-compounded vaginal gel. Although the method is useful to assess the 6 phenolic compounds, some compounds could not be detected in the products.

Key words: Gel, HPLC-UV/DAD, polyphenols, *schinus terebinthifolius* raddi., simultaneous assay, validation

SUMMARY

- A sensitive, selective, accurate and precise reversed-phase HPLC-UV/DAD method for the simultaneous assay of six polyphenols in *S. terebinthifolius* Raddi

Abbreviation used: RP-HPLC-UV/DAD: Reverse Phase High Performance Liquid Chromatograph with Ultraviolet and Diode Array Detector, HPLC: High Performance Liquid Chromatograph, HPLC-UV: High Performance Liquid Chromatograph with Ultraviolet Detector, ANVISA: Brazilian National Health Surveillance Agency, LOD: Limit of detection, LOQ: Limit of quantitation



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INTRODUCTION

Schinus terebinthifolius Raddi, a medicinal plant known in Brazil as 'Aroeira da Praia', has anti-microbial, anti-inflammatory, anti-allergenic, and anti-ulcerogenic properties. In addition, this plant has been used as a febrifuge, analgesic, depurative, as well as to treat urogenital system pathologies.[1-3] Studies have been conducted to evaluate the antimicrobial activity of this species against *S. aureus* and *P. aeruginosa*, a common cause of urinary tract infections.[4,5] According to Amorim and Santos,[6] a vaginal gel prepared from extracts of *S. terebinthifolius* Raddi showed potential for the treatment of bacterial vaginosis, achieving a cure rate of 84% when used by non-pregnant women. These biological properties have been attributed to the presence of phenolic compounds, such as tannins and flavonoids in these extracts.[7-11]

Silva-Corazza *et al.*[12] have described a spectrophotometric method based on the Folin-Ciocalteau reaction for the determination of polyphenols in topical gels containing extracts of *S. terebinthifolius*. Although this method is simple and fast, it does not allow evaluation of each chemical constituent separately, quantifying only total amount of polyphenols. Moreover, the sample preparation step yields a recovery of about 90%.

High-performance liquid chromatography (HPLC) has been successfully applied to analyze plant extracts and products.[13,14] This method has several advantages, such as efficient separation, identification and quantification of similar compounds. However, to the best of our knowledge, no HPLC-UV simultaneous method has been reported to evaluate extracts and products from *S. terebinthifolius*. According to the method described by Carvalho *et al.*[15] only one compound - gallic acid - could be determined and the samples needed to be submitted to pre-treatment steps, where excessive solvent and time were expended. Due to it's porous structure, the monolithics columns have several hydrodynamic advantages comparing to conventional columns. These columns support the use of higher flux with significantly lower pressures than the conventional with the same dimensions. Consequently, it needs

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less analytical time to an efficient separation and lower time to rebalancing the mobile phase between the injections (KELand GUIOCHON, 2002; KRIZMAN *et al.*, 2007). In this context, the aim of the present work was to develop and validate a new simultaneous HPLC-UV/DAD method for the determination of polyphenols that could be present in gels containing *S. terebinthifolius* extracts.

MATERIALS AND METHOD

Collection of plant material

The bark from the stems of *Schinus terebinthifolius Raddi* (Anacardiaceae) was collected in the Atlantic rainforest located in Paraiba, Brazil in august/2010 (coordinates: 651'29.86"S 3529'39.02"O) and identified by the botanist Leonardo Versieux. A voucher specimen was deposited at 'Parque das Dunas' Herbarium, under record number 20. The material was dried in a hot air circulation oven at 45°C ± 2°C for 5 days. The dried bark was ground in a cutting mill (Forlab model FL32) at a high speed.

Chemicals and reagents

HPLC grade acetonitrile and methanol were purchased from JT Baker®. Analytical grade acetic acid, dichloromethane, ethyl acetate, and *n*-butanol were supplied by Synth®. Gallic acid, chlorogenic acid, rutin were obtained from Sigma-Aldrich (Atlasville, South Africa) and catechin, epicatechin, luteolin from Merck (Darmstadt, Germany), all with purity of 97% or greater.Water was purified using a Milli-Q system from Millipore (Milford, MA, USA). Mobile phase was filtered and degassed by passing this through 0.45 µm Nylon filters (Millipore, Bedford, MA) under reduced pressure.

Preparation of the samples

The stock standard solutions were prepared by dissolving 10 mg of each compound in 10 ml of methanol. The volume was completed with the mobile phase (as described in the validation section) to obtain final concentrations of: 1 $mg.ml^{-1}$ for gallic acid, 2.0 $mg.ml^{-1}$ for chlorogenic acid, and 0.4 $mg.ml^{-1}$ for the other phenols.

Hydroalcoholic extract was obtained by maceration of 75 g of the pulverized dried bark in 1000 ml of ethanol at 40% for 5 days at room temperature with occasional shaking. This was done considering the optimal conditions for polyphenol extraction with a yield of 9,3% (n=3) as determined in previous studies (data not published). The analyses were carried out after drying a volume of extract at 50°C, resulting in a dried residual mass of 2.73% ± 0.41, as determined by the Brazilian Pharmacopoeia method. This was followed by re-dissolving in methanol using ultrasound for 30 min, and completing the final volume with the mobile phase to obtain concentrations of between 2.5 and 5.0 $mg.ml^{-1}$. The solutions were filtered through a cellulose acetate filter.

To evaluate the application of the method to gel products containing *S. terebinthifolius* extract, a commercially available vaginal gel (with declared 0.67 ml of extract per gram of gel) and another obtained from a compounding pharmacy (prepared with 0.11 ml of extract per gram of gel) were used. About 5 g of the samples were dissolved in 20 ml of methanol and the final volume completed with mobile phase to obtain concentrations of 0.05 and 0.10 g of $gel.ml^{-1}$. All the samples were sonicated for 30 min in a water bath and filtered through Whatman filter paper (No. 41). Thus,the solutions obtained were filtered through a 0.45 µm Millipore membrane before injection.

Instrumentation

The development of the method was performed on a Shimadzu System consisting of a chromatograph (Shimadzu, Tokyo, Japan), equipped with an LC-10AT pump, a UV/DAD detector (SPD-M20A), a DGU-20A5 degasser, a column oven (CTO-20A), and a SIL-20A auto-injector.

Columns and elution conditions

To obtain reasonable resolutions and to avoid peak tailing, an optimization of the proposed method was performed by using two C18 columns: a conventional C18 *ACE-121-1504* (150 × 4.6 mm; particle size 5 µm) and a monolithic C18 *Chromolith* (100 × 4.6 mm). The mobile phase consisted of a 1:1 (v/v) mixture of acetic acid 2% (component A) and acetonitrile-water-acetic acid at 50:48:2 v/v (component B). For the monolithic column, two different gradient systems were evaluated at a flow rate of 2 $ml.min^{-1}$ as follows:

I - increasing component B concentration from 10% to 30% up to 30 min. and keeping constant to the end

II - starting with B constant at 5% up to 10 min, then increasing to 30% from 10 to 25 min, and keeping constant at 30% to the end. For the conventional column, four gradient systems were tested, where the mobile phase was pumped at a flow rate of 1 $ml.min^{-1}$

III - increasing component B from 10% to 20% up to 4 min, then up to 25% from 4 to 10 min and to 100% from 10 to 30 min, keeping constant to the end

IV - the same procedure as III up to 4 min, then increasing to 24% from 4 to 20 min, and to 100% from 20 to 30 min, keeping constant to the end

V - starting with B constantat 10% up to 25 min, increasing from 10% to 20% up to 30 min, and from 20% to 100% up to 60 min

VI - starting only with component A up to 5 min, at which point 5% of B was introduced, then B was increased from 5% to 25% up to 15 min and from 25% to 100% up to 60 min.

Chromatographic separation was carried out at room temperature (25°C, maintained with air conditioning) and the injection volume was 20 µl.

Data collected with a detector wavelength of 280 nm were used for validation.

Validation

The method was validated according to the guidelines contained in Resolution-RE n 899, ANVISA (Brazilian National Health Surveillance Agency).[16] The parameters evaluated were linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), specificity, robustness, and stability assay. Statistical analyses were performed using the Minitab 15 software package. A probability level of less than 5% ($p < 0.05$) was considered significant.

The specificity was determined by analyzing the chromatographic profile (retention time and resolution) of the samples. To check the presence of co-eluting substances, peak purity was evaluated using a UV/DAD detector. A standard solution containing the compounds studied was compared to the hydroalcoholic extract solution. The spectral profiles of the peaks for each substance were compared with those observed for the peaks obtained at the same retention time for the samples. Purity was evaluated based on the % similarity between the spectra data.

The linearity was evaluated for standard solutions and extract. Samples were prepared as mentioned above. For standard solutions, seven concentrations for each phenolic compound were analysed in triplicate: 25, 30, 35, 40, 45, 50, and 55 $\mu g.ml^{-1}$ for gallic acid; 50, 60, 70, 80, 90, 100, and 110 $\mu g.ml^{-1}$ for chlorogenic acid and 5, 10, 15, 20, 25, 30, and 35 $\mu g.ml^{-1}$ for catechin, epicatechin, rutin, and luteolin. Six solutions

prepared from the extract were analyzed in triplicate at 2.3, 3.0, 3.5, 4.0, 4.5, and 5.0 mg.ml^{-1}.

The accuracy and precision were assessed by performing three replicate assays at three different concentrations of each analyte: 25, 40 and 50 μg.ml^{-1} (gallic acid); 50, 80 and 100 μg.ml^{-1} (chlorogenic acid); 5, 20 and 30 μg.ml^{-1} (catechin, epicatechin, rutin and luteolin). In addition, inter-day precision was determined by analysis of each sample on two different days by two analysts, where the relative standard deviation was calculated.

LOD and LOQ were determined based on the standard deviation of the *y*-intercept, as described in Resolution RE-899, 2003.[16] The robustness of the method was checked by varying: pH of the mobile phase (3.0, 3.5 and 4.0), initial proportion of component B in the mobile phase (from 0 to 5%), column batch (A51497 and A51664), and flow rate (0.9, 1.0 and 1,1 ml.min $^{-1}$). A standard solution was injected for each condition, and the system suitability parameters were then calculated.

The stability of standard and sample solutions was determined by comparing the concentration of each compound in the freshly prepared solutions with levels those after storage at room temperature and at 4°C for 12 h and 30 days, respectively.

RESULTS AND DISCUSSION

Method development

Monolithic columns allow the use of higher flow rates with lower back pressures when compared to conventional columns of the same measurements. Therefore, these columns require shorter assay times for efficient separation, and allow faster column equilibration between runs.[17,18] These characteristics are especially important for long run-time analysis, such as those obtained for complex vegetal samples. However, in the present study, the use of a monolithic column with the proposed gradient systems (I and II) did not provide the adequate separation of some of the components given that co-eluting peaks were verified. Satisfactory interactions between gallic acid and stationary phase were not observed, which may have been due to the high polarity of the former. According to another study, gallic acid is the major phenolic component of *S. terebinthifolius,*[15] which makes the use of these columns unsuitable for analyzing products derived from this species. In addition, the small reduction in run time was insufficient to justify the higher mobile phase consumption.

The peaks of catechin, chlorogenic acid and epicatechin presented poor resolution when analyzed by gradient system III in the conventional column. Infact, this effect may be attributed to co-elutions, a frequently observed problem when complex matrices are analyzed. On the other hand, in gradient system IV, a better resolution of epicatechin was observed, while catechin and chlorogenic acid presented very similar retention times. The compounds of intermediate polarity presented extremely large peaks when method V was tested. It is noteworthy that the catechin peak was *asymmetric* (usually with high tailing) under all experimental conditions. Finally, the best resolution for all peaks and the shortest run time were obtained using the chromatographic conditions described for system VI [Figure 1].

Validation

The results of the validation study are depicted in Table 1. Since all the data were in accordance with the specifications established by Reference RE-899,[16] this method can be considered validated.

As can be seen in Figure 1, complete separation of the analyte peaks was achieved. HPLC-UV/DAD analysis revealed that the peak purity, and similarity concerning the phenolic compounds in the extract, was satisfactory as compared to the isolated compounds, which are considered

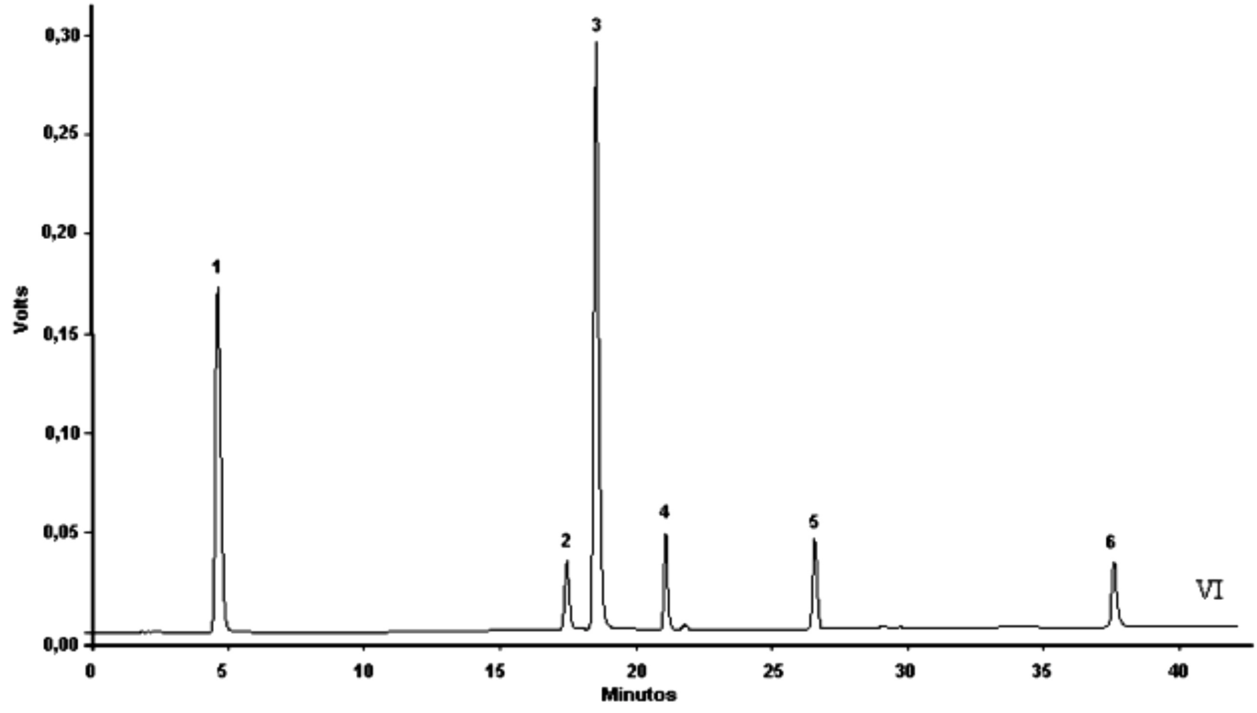

Figure 1: Chromatogram of standard solution analyzed by HPLC, at flow rate of 1 mL.min^{-1}, gradient system: acetic acid 2% from 0 to 5 minutes, addition of 5% acetonitrile-water-acetic acid (50:48:2 v/v) and linear increase up to 25% from 5 to 15 minutes, then up to 100% from 15 to 60 minutes. 1- Gallic acid; 2-Catechin; 3- Chlorogenic acid; 4-Epicatechin; 5-Rutin; 6-Luteolin.

Table 1 : Results obtained in the validation study

Peak/Standard		Linearity			Limits		Accuracy and Precision				
		Range			LOD	LOQ	Day 1			Day 2	
		(µg.mL^{-1})	Equation	r^2	(µg.mL^{-})	(µg.mL^{1})	Theoretical Concentration (µg.mL^{-1})	Found Concentration (µg.mL^{-1})	Recovery (%)	Found Concentration (µg.mL^{-1})	Recovery (%)
1	GallicAcid t_R = 5.11	25 - 55	y = 67075390.5x + 51504	0.9953	2.539	7.693	25.00	25.80	101.90 ± 3.26	26.30	105.31 ± 3.36
							40.00	41.30	103.15 ± 4.07	41.40	105.31 ± 3.36
							50.00	50.30	100.59± 4.19	50.20	100.50 ±1.55
2	Catechin t_R = 18.30	5 - 35	y = 15893771.4x - 11367	0.9974	0.660	2.000	5.00	5.10	102.40 ± 3.41	5.20	104.05 ± 6.07
							20.00	20.00	99.93 ± 2.10	20.10	100.30 ± 3.52
							30.00	30.00	100.07 ± 1.41	30.00	99.95 ± 1.73
3	Chlorogenic Acid t_R = 19.50	50 - 110	y = 33988121.4x + 84991	0.9931	0.630	1.910	50.00	50.10	100.24 ± 4.57	50.40	100.73 ± 2.33
							80.00	79.90	99.87 ± 3.51	81.70	102.16 ± 3.23
							100.00	100.00	99.97 ± 3.22	100.70	100.70 ± 3.79
4	Epicatechin t_R = 21.85	5 - 35	y= 7924602.4x-1117	0.9952	0.336	1.018	5.00	5.20	104.59 ± 1.19	5.00	100.18 ± 1.35
							20.00	21.20	105.84 ± 1.32	21.00	105.14 ± 2.53
							30.00	31.20	104.11 ± 1.15	31.60	105.33 ±1.69
5	Rutin t_R = 27.30	5 - 35	y= 1,6416781.0 x- 3852.6	0.9956	1.421	4.307	5.00	5.20	103.44 ± 2.87	5.10	102.10 ±1.45
							20.00	20.50	102.56 ± 3.12	20.70	103.71 ± 1.56
							30.00	32.30	107.56 ± 1.17	31.60	105.40 ± 1.26
6	Luteolin t_R = 38.63	5 - 35	y= 0488480.09x + 3130.9	0.9958	1.275	3.864	5.00	5.00	100.96 ± 3.56	5.10	102.89 ±1.23
							20.00	20.10	100.61 ± 2.17	20.40	101.97 ± 3.94
							30.00	30.50	101.77 ± 2.47	30.30	101.06 ± 3.28

Theoretic = theoretical, conc. = concentration, t_R = retention time

to have high spectral purity (minimum value obtained for purity was 99.01% for luteolin and 99.02% for epicatechin similarity). These results demonstrated the absence of co-eluting substances, confirming that the method is adequate for analyzing gallic acid, catechin, chlorogenic acid, epicatechin, rutin, and luteolin in *S. terebinthifolius* products.

The analytical curves were linear over a wide concentration range and the correlation coefficients were greater than 0.9931 when standard solutions were used. Regarding extract solutions, the method did not have good linearity concerning catechin and epicatechin, with correlation coefficients of 0.9538 and 0.9439, respectively. Therefore, this method is indicated only for identification of these compounds but not for quantification.

In addition, LOD and LOQ values were superior to those described in other methods,[19-24] showing the lower sensitivity of the proposed method. We hypothesize that the relatively low sensitivity could have been due to the choice of a single wavelength for all compounds studied, whose value does not correspond to the maximum wavelength of each compound. To improve sensitivity, selected wavelengths should be applied during analysis to obtain the maximum absorbance for each compound.

At a significance level of 5%, no difference was observed between the results obtained on first and second days of analysis. Considering a maximum variance of 5%, standard solutions stored at room temperature can be analyzed for up to 12 h. When the solutions were kept at 4°C, the peak corresponding to luteolin had a significant decrease after 10 days of storage. On the other hand, samples presented lower stability, especially concerning catechin and epicatechin concentrations at room temperature. However, akin to standard solutions, all samples stored at 4°C for 10 days proved stable except for luteolin. Gallic acid standard solutions, the major component of *S. terebinthifolius* extract, was stable under both conditions.

According to the system suitability test, this method can be considered robust for the flow rate, initial proportion of phase B and column batch variations, as all parameters met the USP recommendations. The pH of the mobile phase seems to be a more critical factor, where peaks 1 and 3 had caudal asymmetry and high values for tailing. Changing the pH of the mobile phase is usually necessary to avoid ionization of the hydroxyl groups present in phenolic compounds. In fact, more efficient separations and symmetrical peaks are achieved when the sample diluents and mobile phase are acidified.[15]

Application of the proposed method

As shown in Figure 2, gallic acid (peak 1) can be considered the major component in samples of *S. terebinthifolius* gels and hydroalcoholic extract. However, chlorogenic acid was found at a much lower concentration in the gels than in the extract.

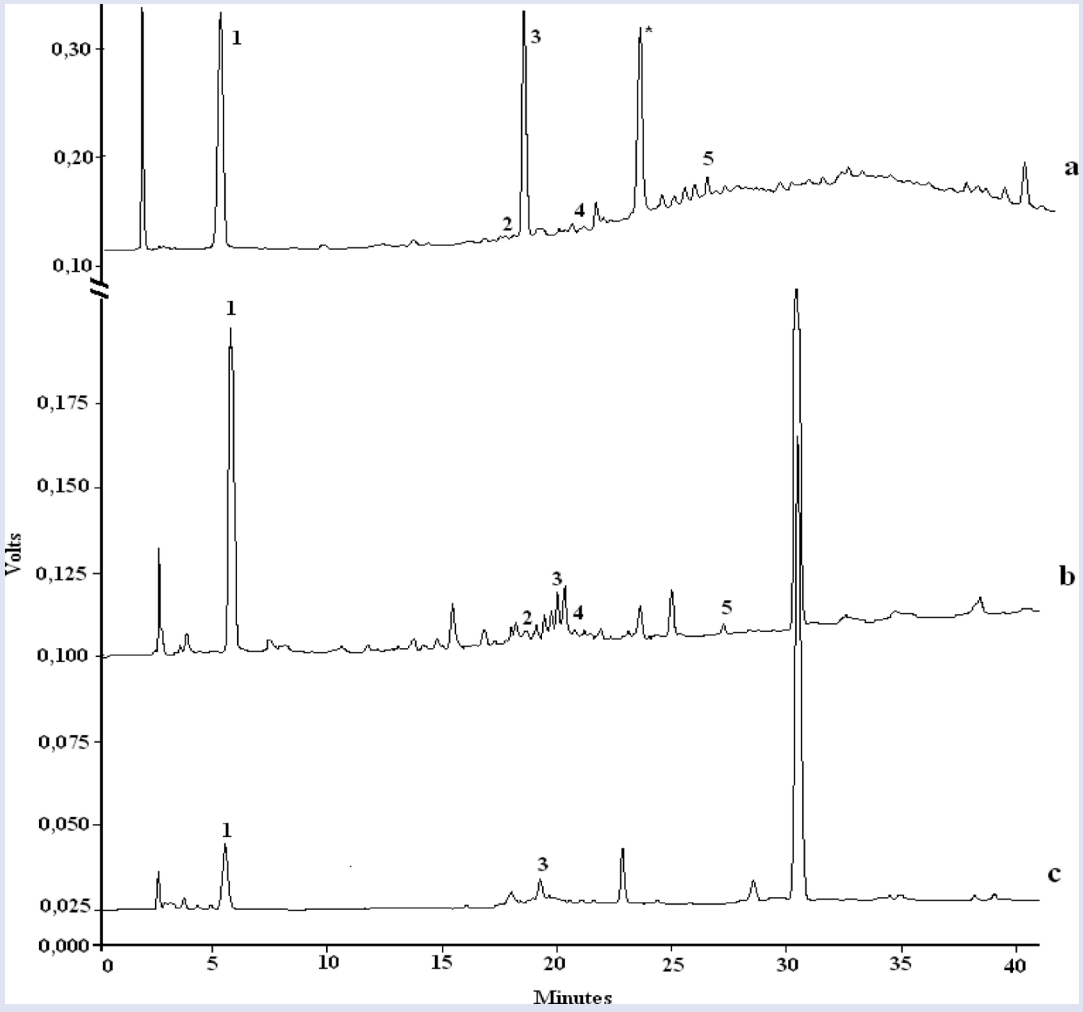

Figure 2: Chromatograms of analyzed products: Hydroalcoholic extract (a), commercially available (b) and pharmacy-compounded (c) gels. 1-Gallic acid; 2-Catechin; 3-Chlorogenic acid; 4-Epicatechin; 5- Rutin.

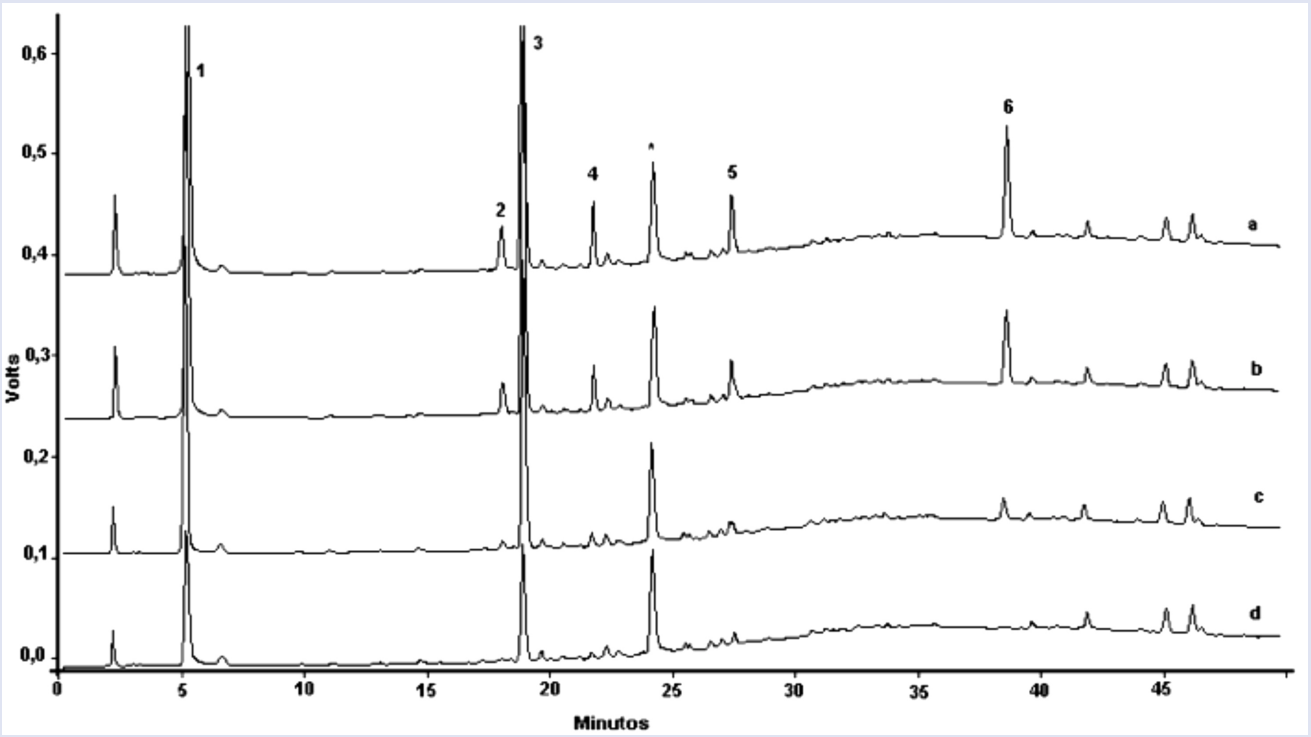

Figure 3: Chromatogram of hydroalcoholic extract (**d**) added of standard solution 1- Gallic acid; 2-Catechin; 3- Chlorogenic acid; 4-Epicatechin; 5-Rutin; 6-Luteolin in high (**a**), medium (**b**) and lower (**c**) concentration.

Pharmacy-compounded gels of *S. terebinthifolius* are usually prepared at a concentration of 0.10 ml of extract/g of gel, which is about 6 times lower than the concentration observed for commercially available gels. It appears that the substantial amount of excipients typically found in pharmacy-compounded products may have interfered with the dissolution of its components, making it difficult to achieve the same concentration as the commercially-available sample. In fact, the presence of a peak at 30.9 min of analysis in the products can be assigned to some of the excipients present in the gels [Figure 2].

Since the phenolic compounds were present at very low concentrations in these samples, its determination became more challenging. The presence of gallic acid, chlorogenic acid and rutin in the extract was confirmed by increased peak areas after coinjection with the respective standards. Thus, from the 6 substances used in this study, gallic acid, chlorogenic acid and rutin can be quantified in the extract [Figure 3]. Although these compounds could be identified, some compounds were not detected, especially in the compounded gel, probably due to their low concentrations in the sample.

Therefore, this raises the question as to whether the use of these pharmacy-compounded *S. terebinthifolius* gel with such low concentrations of phenolic compounds can still produce the expected clinical responses. This finding shows the importance of determining the concentration of this species in the gels to confirm its effectiveness.

CONCLUSION

In conclusion, besides being simple and specific, this method showed reasonable accuracy, precision and linearity. It did not involve laborious time-consuming sample preparations and can therefore be considered suitable for the routine quantitative analysis of gallic acid, catechin, chlorogenic acid, epicatechin, rutin, and luteolin in gels containing *S. terebinthifolius*, as well as in its extracts.

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Nil

Conflicts of interest

There are no conflicts of interest.

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