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Original Article

Development of standardized extractive solution from *Lippia sidoides* by factorial design and their redox active profile

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ABSTRACT

The aim of this study was to evaluate the influences of variables of preparation on total flavonoids content from extractive solution of *Lippia sidoides* Cham., Verbenaceae. Thus a 2^3 factorial design was used to study the importance of plant proportion, the extraction method and solvent on the extraction of flavonoid. The methodology of determination of chemicals in factorial design was validated according to the parameters required by Brazilian Health Agency. The extraction solution was selected through a full factorial design where the best conditions to achieve the highest content of flavonoids were: 7.5% (w/v) of plant with ethanol 50% (v/v) as solvent. The polyphenols content was determined by LC method and its relationship with the antioxidant and free radical scavenging activities was evaluated. The free radical scavenging activities and antioxidant potentials were determined for different concentrations using various *in vitro* models. Our results indicate that extracts exhibited a significant dose-dependent antioxidant effect as evaluated by TRAP/TAR assays. Besides, we observed an antioxidant activity against hydroxyl radicals and nitric oxide, and protection against lipid peroxidation *in vitro*. Our results suggest that the extract presents significant *in vitro* antioxidant potential indicating promising perspectives for its use as pharmaceutical/or food additive.

Introduction

Lippia sidoides Cham., Verbenaceae, popularly known as "alecrim-pimenta", is a typical shrub commonly growing in the Northeast of Brazil. This species produces an essential oil rich in thymol and carvacrol, which has a potent antimicrobial activity against fungi and bacteria (Lemos et al., 1990; Lacoste et al., 1996). The hydro-alcoholic extracts of this plant are largely used for treatment of skin wounds, as a mouth antiseptic and in liquid soap preparations to treat and prevent general fungal infections of the body (Matos, 2000).

In this way, those groups of substances can be successfully used as chemicals markers which can assist in the studies of standardization of extractive solutions from medicinal plants.

However, validated quality control methods need to be developed in order to comply with regulatory requirements, if some plant is to be used as raw material by the pharmaceutical industry; the absence of those studies hampers the reproducibility of the extractives qualities, which could affect the efficiency and safety. Thus, the standardization of plant extractive solutions should be the first step during the technological development of phytopharmaceuticals. The influence of several parameters such as extractive method and technology, type and concentration of solvent, as well as plant concentration, and their influence in the physical-chemical properties of the extractive solutions should be evaluated and quantified (Audi et al., 2001; Cunha et al., 2009).

The factorial design is a statistic tool used to screening and/or optimization process, with rapid and economic way, and maximization the quality of final products. Besides, the statistical analysis award results are reliable (Myers and Montgomery, 1995; Montgomery, 1997). Thus, it is possible to rank each independent variable according to its significance on the studied responses. Therefore, with reduced time and experimental effort, it may be

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possible to choice the extractive conditions that are able to produce the maximum experimental response (Myers and Montgomery, 1995; Montgomery, 1997; Canteri-Schemin et al., 2005; Soares et al., 2005).

The aim of this work was to use the factorial design to evaluate the effect of the plant proportion, solvent type, concentration and the extraction method on the total flavonoids content in extractives solutions from *L. sidoides*. Additionally, the aim was also to evaluate the *in vitro* antioxidant activity of the optimized extractive solution.

Materials and methods

Plant material

Aerial parts of *Lippia sidoides* Cham., Verbenaceae, were collected in the garden of medicinal plants of the Federal University of Sergipe (São Cristóvão, Brazil) in May 2009 and was identified by Prof. Ana Paula N. Prata who is a plant taxonomist from the Department of Biology. A voucher specimen (ASE 2626) has been deposited in the Herbarium of Department of Biology. The plant material after harvesting was subjected to drying process in a circulating air at a temperature of $40 \pm 2\,^{\circ}C$ until stabilization of residual moisture. After drying the plant material was manually selected, subjected to grinding in a grinder of knives, weighed, sampled and was characterized through analysis of moisture content, granulometric analysis using sieve of 150, 250, 355, 500, 600, 710 and 1000 μm, loss of drying by gravimetric method and determination of extractive content.

Evaluation of analytic methodology by spectrophotometry

Through the technique of determination without acid hydrolysis (Petry et al., 1998; Silva et al., 2009; Marques et al., 2012), the sample of raw material was subjected to extraction under reflux (7.5%, w/v) and was held in a dilution of 3:50 ml of stock solution in water distilled; then, in flasks of 20 ml aliquots were added to 3.2 ml of the diluted solution and 1.6 ml of $AlCl_3$ (2.5% and 7.5%) in order to assess what would be the ideal concentration of $AlCl_3$ necessary for chelating flavonoids like quercetin found in the extractive solution of *L. sidoides*. Then, the balloons were filled with ethanol (40%) and 30 min after the readings were performed in a spectrophotometer at 423 nm (maximum of $AlCl_3$-quercetin complexed). Given the optimal concentration of $AlCl_3$ to complexation was performed a reading of scanning the extractive solution before and after complexation which comprised a range of 200–800 nm in which it was intended to strengthen the displacement of bathochromic flavonoid solution.

Validation methodology

A methodology to determine and quantify the flavonoids in raw material must be validated considering the following parameters: specificity, linearity, precision, accuracy and robustness (Anvisa, 2003).

The statistic analysis and experimental design

The experimental matrix was a 2^3 factorial design and it was used to evaluate the influence of plant proportions (5.0/7.0%; w/v), solvent type (water/ethanol 50%) and the extraction method (infusion/decoction) on the efficiency of the extraction of flavonoids. The experiments were performed in triplicate, and the total flavonoids content (TFC) was used as responses. The statistical analysis of the factorial design was performed using the software Statistica® 6.0 (StatSoft, USA). The experimental data were analyzed by ANOVA and *t*-test for standardized effects (Myers and Montgomery, 1995).

HPLC analysis of extractive solution

Preparation of L. sidoides *extracts*

Dry extract (100 ml) obtained by decoction (7.5%, w/v in 15 min) was diluted with methanol/milli-Q water (50:50, v/v) to achieve a concentration of 10 μg/ml. For injection in the HPLC, the solutions were filtered in a 0.44 μm (regenerated cellulose) membrane.

Analytical and preparative HPLC

The HPLC analysis was performed on a Shimadzu system consisting of a degasser DGU-20A3, a SIL-20A autosampler, two LC-20AD pumps and a SPDM20Avp photodiode array detector (DAD), coupled with a CBM20A interface. Before injection into the HPLC, the solutions were filtered in a 0.44 μm (regenerated cellulose) membrane. The analytical method employed a linear gradient system which consisted of (A) acetic acid:water 1.0% (v/v); (B) methanol. The chromatographic separation has been performed using a Phenomenex Luna C18 analytical column 250 mm × 4.6 mm (5 mm particle size). The flow rate was 0.6 ml/min and the injection volume was 20 μl. The mobile phase consisted of gradient of water and methanol starting with 10% B during 20 min; 40–45% B during 5 min; 45–60% B during 10 min; 60–75% B during 25 min total time of 60 min. Photodiode array detector was set at 254 nm for acquiring chromatograms. The analysis of peaks was based in accordance with the retention time of the standard substance and mass spectrum (MS/MS). Mass spectrometric analysis was performed on a Brucker mass spectrometer fitted with ion trap ionization source. The negative ion mode [*m/z* M−H] was used for all compounds. Ion trap ionization was operated in MRM and conditions were: nebulizer pressure: 40 psi; dry gas flow: 9 l/min; drying gas temperature: 300 °C; flow rate: 200 μl/min.

Redox active profile

Total antioxidant potential (TRAP) and total antioxidant reactivity (TAR)

TRAP/TAR was determined by measuring the chemiluminescence (CL) intensity of luminol induced by 2,2′-azobis (2-amidinopropan) dihydrochloride (AAPH) (Lissi et al., 1995). The background CL was measured by adding AAPH and luminol. Then, the samples (OELS from 1 ng ml^{-1} to 1 mg ml^{-1}) were added, and the CL was measured in a liquid scintillator counter. The last count before the addition samples was considered as 100%. Graphs were obtained by plotting percentage of counts per min (% cpm) *versus* time (s). The AUC (TRAP assay) was calculated using GraphPad Prism software. The TAR was calculated as the ratio of light intensity in absence of samples (Io)/light intensity right after OELS addition (I).

TBARS (Thiobarbituric Acid Reactive Species)

TBARS assay was employed to quantify lipid peroxidation and an adapted TBARS method was used to measure the antioxidant capacity of OEHP using egg yolk homogenate as lipid rich substrate (Silva et al., 2007). Briefly, egg yolk was homogenized (1%, w/v) in phosphate buffer (pH 7.4), 1 ml of homogenate was sonicated and then homogenized with 0.1 ml of OELS at different concentrations. Lipid peroxidation was induced by addition of 0.1 ml of AAPH solution (0.12 M). Samples were centrifuged with trichloroacetic acid

at 1200 *g* for 10 min. An aliquot of 0.5 ml from the supernatant was mixed with 0.5 ml TBA and heated at 95 °C for 30 min. After cooling, samples absorbance was measured using a spectrophotometer at 532 nm. The results were expressed as a percentage of TBARS formed by AAPH alone (induced control).

Hydroxyl radical ($^{\bullet}OH^{-}$) scavenging assay

Hydroxyl radicals were generated by a Fenton system ($FeSO_2$-H_2O_2). When exposed to hydroxyl radicals, the sugar deoxyribose is degraded to malonaldehyde (MDA), which generates a pink chromogen on heating with TBA at low pH. The method for determining the scavenging on hydroxyl radicals was performed according to a previously described procedure.

Nitric oxide (NO) scavenging assay

Nitric oxide was generated from spontaneous decomposition of sodium nitroprusside (SNP) in the phosphate buffer (pH 7.4). Once generated NO interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction. The reaction mixture containing SNP in phosphate buffer and OELS at different concentrations were incubated at 37 °C for 1 h. An aliquot was taken and homogenized with Griess reagent. The absorbance of chromophore was measured at 540 nm. Percent inhibition of nitric oxide generated was measured by comparing the absorbance values of negative controls (SNP and vehicle) and assay preparations.

Results and discussion

Characterization of the plant material

The plant material of *L. sidoides* used in this research presented particle mean diameter of 325 μm, loss on drying of 11.25% and extractive content of 19.83%. These proprieties are important for standardization of extractive process, since particles mean diameter influences the extraction efficiency and the loss on drying is important to conservation of raw material (List and Schimdt, 1989).

Development of analytic methodology by spectrophotometry

The aluminum cation forms stable complexes with flavonoids, which leads to a deviation of the peak absorption to longer wavelength. Thus it is possible to determine the amount of flavonoids, avoiding the interference of other phenolic substances, mainly phenolic acids, which invariably accompany the flavonoids in plant tissues. This reading is done in spectrophotometer at 425 nm, using aluminum chloride to 2%. There was no statistically significant difference in amplitude of the peak absorption or at the time that this point has been reached regarding the concentration of $AlCl_3$ was used.

Validation of the methodology

According to Brazilian Health Agency (Anvisa, 2003), the purpose of validation is to demonstrate that the method is suitable for their intended purpose, and to identify qualitative, semi-quantitative and/or quantities of drugs and other substances in pharmaceuticals. Thus, the method is validated when has the specificity, linearity, precision, accuracy and robustness within the parameters required.

The specificity of method was demonstrated across the spectrum scanning of the extractive solution from *L. sidoides* (7.5%, w/v) and the standard quercetin (50.0 μg/ml), both complexed with aluminum chloride (2.5%), only one peak being shown of maximum absorption at 423 nm for quercetin and other at 405 nm for the extractive solution (Fig. 1).

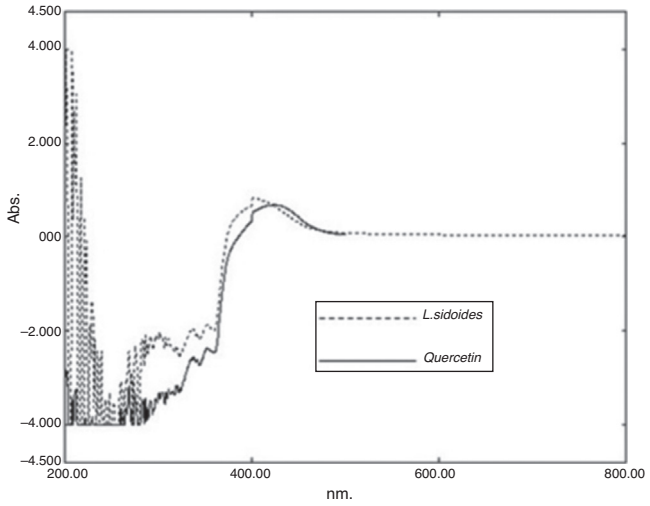

Fig. 1. Spectrum scanning of the extractive solution from *L. sidoides* and the quercetin standard.

The linearity was obtained through of the calibration curve of quercetin diluted in ethanol (40%). This curve was made with five different concentrations: 10, 20, 30, 40 and 50 μg/ml. The parameter linearity is very important because it demonstrates that the results obtained are directly proportional to the concentration of analyte in the sample, within a specified range.

The test was made in triplicate and the results of the statistical analysis are showed in Table 1. The equation of the line obtained was $y = 0.01467x + 0.3062$, the accuracy of the determinations was between 102.10 and 101.11% (Table 1) and the determination coefficient obtained was greater than 0.99 ($r^2 = 0.9992$) which is within the parameters of existing legislation (Anvisa, 2003).

The precision was obtained at two levels: repeatability, which evaluated the concordance between the results within a short period of time with the same analyst and same instrumentation. This level was carried in triplicate with six determinations of 100% of the concentration of the test; and the second level was the intermediate precision, which analyzes the correlation between the results from the same laboratory, obtained in different days with different analysts performed in duplicate with six determinations. The results of the statistical analysis of the precision test are shown in Table 2.

The accuracy obtained as indicated by the resolution for this test was thus applied in the analytical methodology proposed in the analysis of a substance of known purity; in low (22.5 μg/ml), medium (45 μg/ml) and high (65 μg/ml) concentration according to the minimum and maximum values on the curve all the results are shown in Table 3.

The robustness was evaluated by variation of temperature between 25 and 35 °C and the solution concentration of aluminum

Table 1
Results of the statistical analysis of the linearity test.

TC	*C*	SD	RSD	*A*
10	10.2152	1.723	1.68	102.10
20	20.4642	2.351	1.15	102.32
30	30.6136	2.093	0.68	101.65
40	40.6821	1.254	0.28	101.90
50	50.5461	2.372	0.46	101.11

TC (μg/ml), theoretical concentration; *C* (μg/ml), mean concentration of three determinations; SD, standard deviation; RSD (%), relative standard deviation; *A* (%), accuracy.

Table 2
Results of the statistical analysis of the precision test.

Test		TC	*C*	SD	RSD	*A*
Repeatability		50.0	50.47	2.45	1.09	99.59
		50.0	50.91	1.75	0.38	100.32
		50.0	49.97	2.12	0.32	100.21
Intermediate precision	Analyst 1	44.0	44.05	2.11	0.47	100.76
	Analyst 2	44.0	44.16	2.09	0.47	100.32

TC (μg/ml), theoretical concentration; *C* (μg/ml), mean concentration of three determinations; SD, standard deviation; RSD (%), relative standard deviation; *A* (%), accuracy.

Table 3
Result of the statistical analysis of the accuracy test.

Test	CL	TC	*C*	SD	RSD	*A*
Accuracy	Low	22.5	22.475	2.45	1.09	99.89
	Medium	45.0	44.912	1.75	0.38	99.80
	High	65.0	64.978	2.12	0.32	99.95

CL, concentration level; TC (μg/ml), theoretical concentration; *C* (μg/ml), mean concentration of three determinations; SD, standard deviation; RSD (%), relative standard deviation; *A* (%), accuracy.

chloride in method between 2.5 and 7.5%. The analyses were done in triplicate. The method was robust for the variation of temperature and solution concentration of aluminum chloride.

Experimental design

The results for the total flavonoids content of each extractive solution are presented in Table 4. The standardized effects of each main factor as well as respective interactions are presented in Table 5. Regarding the statistical analysis of experimental data, the most important effect could be attributed to the factor Solvent, which provides more efficient extractive procedure when ethanol 50% (v/v) was used as solvent. According to *t*-test, the second main factor was the drug proportion. No statistically significant effect could be imputed to extraction methods (infusion/decoction). On the other hand, the interactions of extraction method were able to provide improvements on the extractive efficiency either with Solvent or Plant proportion. The expected improvements in the yield of TFC due to increasing drug amount as well as the positive effect of ethanol:water mixture undergo extra enhanced effect derived from method of extraction. Although no significant effects were observed for the Method of Extraction, the influence of maintaining the temperature by decoction seems to be essential to promote better wettability of the raw material and higher diffusivity of the solvent.

Separation polyphenols by HPLC

Photodiode array detector was set at 254 nm for acquiring chromatograms. The chromatogram *L. sidoides* extract showed three major peaks well separated: P1 (R.T. 21 min), P2 (R.T. 34 min) and P3 (R.T. 41 min). P1 was identified as chlorogenic acid (Fig. 2). P2 and P3 were not identified but exhibited UV spectra pattern of caffeoylquinic acid derivatives, indicating that this class of polyphenols are the major constituents in the extract. P1 had its mass spectrum analysis and Showed MS 353 *m/z* and MS^2 191 *m/z* (chlorogenic acid molecule).

Table 5
Statistical analysis of experimental data: standardized effects (main factors and interactions).

	Standardized effect	*t*-Test	ANOVA (*F*)
Mean/interc.	14.64	53.11*	–
(1) Method	1.014	1.84	3.38
(2) Plant (%)	2.69	4.88*	23.85*
(3) Solvent	4.37	7.92*	62.67*
1 by 2	1.33	2.42*	5.83*
1 by 3	1.41	2.57*	6.58*
2 by 3	1.00	1.82	6.30

* $\alpha = 0.05$.

Redox active profile

The TRAP and TAR methods are widely employed to estimate the general antioxidant capacity of samples *in vitro*. Thus, the general antioxidant potential of extract from *L. sidoides* was first evaluated by the TRAP/TAR assays. The TRAP/TAR assays indicate that optimized extractive solution of *L. sidoides* (OELS) presents a significant antioxidant activity at the all concentrations studied (Fig. 3).

Lipid peroxidation (LPO) has been defined as the biological damage caused by free radicals that are formed under oxidative stress (Zin et al., 2002). Several plant extracts have been shown to inhibit LPO as measured by the levels of TBARS. The lipids in membrane are continuously subjected to oxidant challenges. Oxidant induced abstraction of a hydrogen atom from an unsaturated fatty acyl chain of membrane lipids initiates the process of LPO, which propagates as a chain reaction. In the process, cyclic peroxides, lipid peroxides and cyclic end peroxides are generated, which ultimately are fragmented into aldehydes like MDA. Similarly, the OELS were able to prevent lipoperoxidation induced by AAPH *in vitro* in a lipid-enriched system (Fig. 4). The OELS at 100 ng ml^{-1} to 1 mg ml^{-1} showed antioxidant activity against hydroxyl radicals (Fig. 5). To determine the ability of extract to act as a reactive nitrogen species (RNS) scavenger, we evaluated the NO-scavenging activity by the Griess method and OELS at 100 ng ml^{-1} to 1 mg ml^{-1} showed a significant ($p < 0.05$) NO-scavenging activity (Fig. 6).

The results found in this study are in agreement with both observations and suggest a direct correlation between antioxidant activity and polyphenol content. It is probable that the active principle responsible by the redox activity in this work is the polyphenol content. Several studies have shown that the redox activity associated with natural antioxidants is attributed to the total content

Table 4
Matrix of the factorial design 2^3.

Exp	Coded variable			Natural variable			TFT (μg/ml)
	Method	Plant	Solvent	Method	Plant (w/v)	Solvent	
1	+1	+1	−1	Decoction	7.5	Water	13.043
2	+1	+1	+1	Decoction	7.5	EtOH 50%	21.280
3	+1	−1	+1	Decoction	5.0	EtOH 50%	14.798
4	+1	−1	−1	Decoction	5.0	Water	11.476
5	−1	+1	−1	Infusion	7.5	Water	13.568
6	−1	+1	+1	Infusion	7.5	EtOH 50%	16.064
7	−1	−1	+1	Infusion	5.0	EtOH 50%	15.157
8	−1	−1	−1	Infusion	5.0	Water	11.752

Exp., experiment; DR, dry residue; TTC, total tannin content; EE, extractive efficiency.

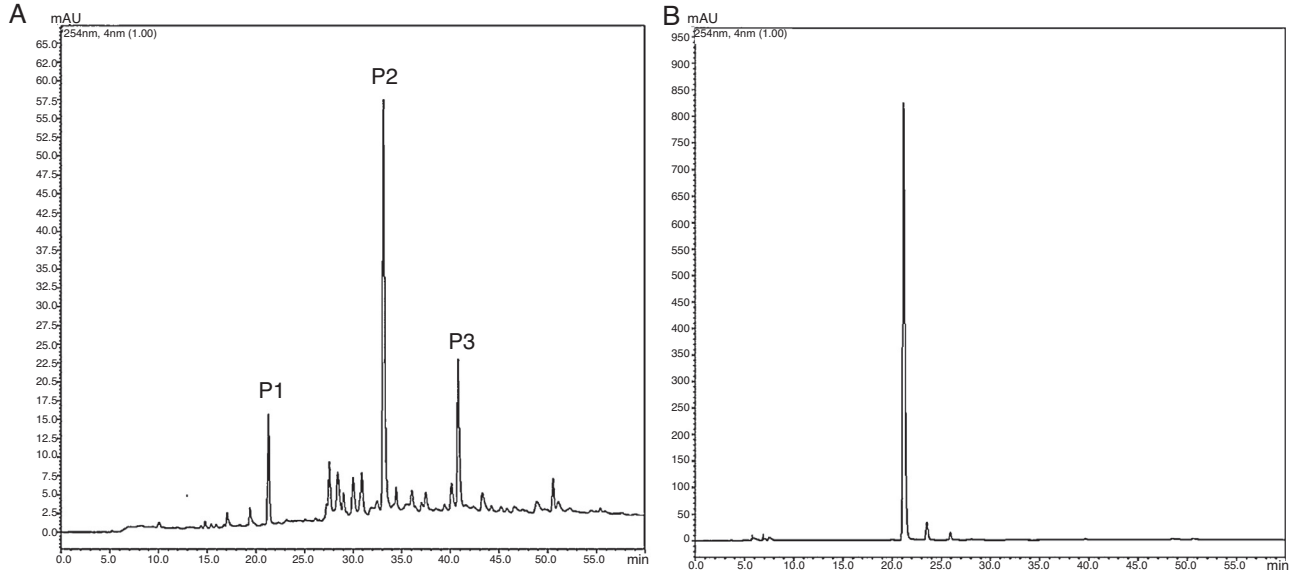

Fig. 2. (A) Chromatogram of extractive solution of *L. sidoides*. (B) Chromatogram of the reference substance (chlorogenic acid).

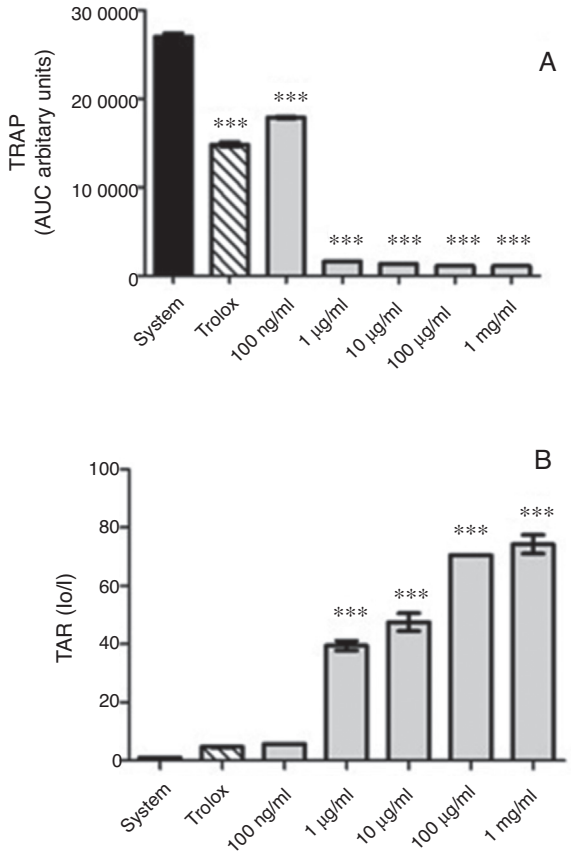

Fig. 3. (A) Total Radical-Trapping Antioxidant Parameter (TRAP) at different concentrations. Values represent mean ± S.E.D., experiments in triplicate, ***$p < 0.001$ different from system and trolox (ANOVA followed by Tukey). (B) The total antioxidant reactivity (TAR) was calculated as the ratio of light intensity in absence of samples expressed as percent of inhibition (I_0/I). Values represent mean ± S.E.D., experiments in triplicate, ***$p < 0.001$ different from system and trolox (ANOVA followed by Tukey).

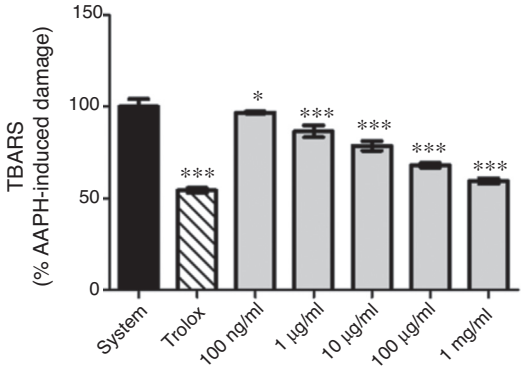

Fig. 4. Thiobarbituric Acid Reactive Species (TBARS) was evaluated from *L. sidoides* (100 ng ml^{-1} to 1 mg ml^{-1}). Values represent mean ± S.E.D., experiments in triplicate, ANOVA followed by Tukey, *$p < 0.05$; ***$p < 0.001$ different from system.

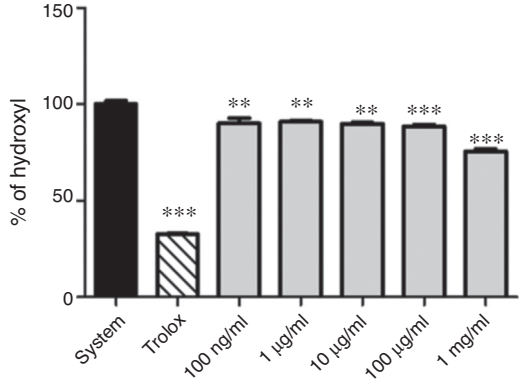

Fig. 5. Hydroxyl radical-scavenging activity from *L. sidoides* (100 ng ml^{-1} to 1 mg ml^{-1}). Values represent mean ± S.E.D., experiments in triplicate, ANOVA followed by Tukey, ***$p < 0.001$ (anti-oxidant) different from system.

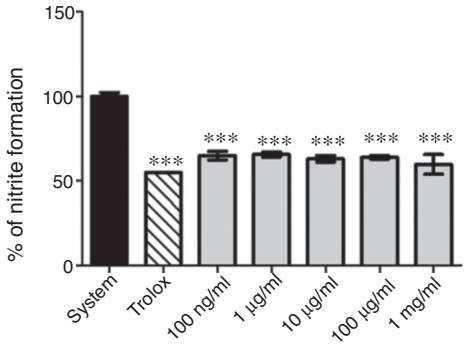

Fig. 6. Nitric oxide (NO) scavenging activity from *L. sidoides* (100 ng ml^{-1} to 1 mg ml^{-1}). Values represent mean ± S.E.D., experiments in triplicate, ANOVA followed by Tukey $^{*}p < 0.05$; $^{***}p < 0.001$ different from system (SNP).

of phenolic compounds (Halliwell, 2008; Rice-Evans et al., 1997; Scalbert et al., 2005; Serafini et al., 2011).

Conclusions

The analysis of data from this study confirmed the ability of the developed method for evaluating the polyphenol content in extracts of *L. sidoides* and the analysis of factorial design showed that the optimum condition to prepare the extractive solutions with maximum total flavonoid content, found extraction by decoction, using 7.5% (w/v) of plant and ethanol 50% (v/v) as the extractive solvent. Concluding, the date presented herein indicates that the *L. sidoides* extract have *in vitro* antioxidant activity and should be considered as new sources of natural antioxidants jointly with other phenolic rich plants. Further studies are needed to examine the potential use of these extracts in the prevention or treatment of pathologies where oxidative stress seems to play an important role.

Authors' contributions

BSL and CSR contributed with chromatographic analysis, factorial design and writing of the manuscript. JPAS, TKR and CASS contributed with the redox active profile. MRS, LALS, LJQJ, JCFM, DPG, AASA and FAS designed the study, supervised the laboratory work, contributed to critical reading of the manuscript, writing of the manuscript and final editing of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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