Industrial Crops and Products 78 (2015) 124–130

Contents lists available at ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop

Optimization and standardization of extraction method from *Lippia origanoides* H.B.K.: Focus on potential anti-hypertensive applications

Angélica G. Coelho[a], José S. Lima Neto[a], Arkellau K.S. Moura[b], Taciana Oliveira de Sousa[b], Ilmara C.P.S. Morais[c], Gabriela D. Carvalho[c], Francisco Valmor M. Cunha[c], Maria das Graças F. Medeiros[a], Eilika A.F. Vasconcelos[a], Aldeídia P. Oliveira[c], Daniel D.R. Arcanjo[c], Lívio C.C. Nunes[a], Antônia M.G.L. Citó[a,b,*]

[a] Programa de Pós-graduação em Ciências Farmacêuticas, Universidade Federal do Piauí, CEP 64049-550 Teresina, PI, Brazil
[b] Departamento de Química, Universidade Federal do Piauí, CEP 64049-550 Teresina, PI, Brazil
[c] Núcleo de Pesquisas em Plantas Medicinais, Universidade Federal do Piauí, CEP 64049-550 Teresina, PI, Brazil

ARTICLE INFO

Article history:
Received 17 July 2015
Received in revised form 3 October 2015
Accepted 13 October 2015
Available online 16 November 2015

Keywords:
Hypertension
Lippia origanoides
Maceration
Naringenin
Standardization
UFLC

ABSTRACT

Lippia origanoides H.B.K. (Verbenaceae) is a medicinal plant used in traditional medical practices for treatment of respiratory and gastrointestinal diseases. Based on previous reports regarding cardiovascular effects induced by *L. origanoides*, this work aims to develop a standardized extract from *L. origanoides* aerial parts (Lo-HAE) and evaluate its hypotensive effect on mean arterial pressure in rats. Eight extraction systems were prepared by varying the ethanol/water ratio, sonication and time of extraction. The chromatographic profile and the determination of the flavonoid naringenin were performed by Ultra-fast Liquid Chromatography (UFLC) with UV detection at 290 nm. The extraction method for Lo-HAE was standardized considering the best extraction yield under 1:1 (v/v) ethanol/water ratio. Naringenin is the major compound of Lo-HAE, and then it was confirmed as the promising biomarker for Lo-HAE control assessment. For pharmacological studies, the acute oral toxicological assessment of Lo-HAE in female Wistar rats was performed. The Lo-HAE-induced hypotensive effect was evaluated by direct measure of pulse pressure after intravenous administration (12.5, 25 and 50 mg/kg), as well as indirect measure of blood pressure after oral administration (100 mg/kg and 200 mg/kg) in female Wistar rats. A marked decrease of mean arterial pressure was observed for Lo-HAE after both administration routes. In addition, no sign of either clinical or behavioral alterations was observed, as well as on the rats' body weight. The Lo-HAE demonstrates safe pharmacological potential for development of herbal medicines in the treatment of hypertension.

1. Introduction

According to the World Health Organization (WHO), cardiovascular diseases cause 17 million deaths per year, 9.4 million correlated with hypertension. This pathology is responsible for 45% of deaths in heart diseases and 51% of death cases in cerebrovascular diseases. The prevalence of hypertension in America is 35% and in Brazil it is the number one cause of death. The Brazilian Health Ministry, through National Policy of Medicinal Plants and Phytotherapics, improved the financial sources for discovery of new active compounds in medicinal plants to decrease the number of cases of hypertension in the country (Brazil, 2006).

The *Lippia origanoides* H.B.K. is a honey plant from the Verbenaceae family which is native to Central America and northwestern South America. The term "origanoides" is attributed from the characteristic oregano-similar aroma, and this species is used as a condiment or as a medicinal plant in the treatment of respiratory and gastrointestinal diseases, as well as a natural antiseptic. Several species from *Lippia* genus are able to induce a hypotensive

* Corresponding author. Current address: Universidade Federal do Piauí, Campus Ministro Petrônio Portella, Departamento de Química, SG-02, Ininga, CEP 64049-550 Teresina, PI, Brazil. Fax: +55 86 3215 5840.
E-mail addresses: angelicacoelho13@gmail.com (A.G. Coelho), limaneto5@gmail.com (J.S. Lima Neto), arkellaukenned@gmail.com (A.K.S. Moura), tacinhasousa@hotmail.com (T.O.d. Sousa), ilmara.cecilia@hotmail.com (I.C.P.S. Morais), dantas.c_gabi@hotmail.com (G.D. Carvalho), orfeuyeuridice@gmail.com (F.V.M. Cunha), mgfmedeiros@hotmail.com (M.d.G.F. Medeiros), eafvasconcelos@hotmail.com (E.A.F. Vasconcelos), aldeidia@gmail.com (A.P. Oliveira), daniel.arcanjo@ufpi.edu.br (D.D.R. Arcanjo), liviocesar@hotmail.com (L.C.C. Nunes), gracacito@gmail.com (A.M.G.L. Citó).

http://dx.doi.org/10.1016/j.indcrop.2015.10.033
0926-6690/

effect as well as being applied in traditional medical practices for the treatment of hypertension (Guerrero et al., 2002; Pascual et al., 2001).

The *L. origanoides* is also considered a promising source of bioactive substances. The medicinal potential of this species can be assigned to different groups of substances, especially due to its high concentration and variety of flavonoids. The flavanones naringenin and pinocembrin and the flavones quercetin and luteolin have been attributed to be the lead compounds which are responsible for the medicinal characteristics of this species (Stashenko et al., 2013). Moreover, naringenin has been reported as the major compound of a best antimicrobial fraction from *L. origanoides* ethanol extract (Barreto et al., 2014).

In this sense, the chromatographic profile and quantitative analysis of biomarkers is required for the production of herbal medicines from vegetable raw materials. Then, extraction systems can be effectively optimized by techniques that involve some underlying factors which have influence in the characteristics of plant extracts, such as: particle size, the solvent polarity of the medium acidity, agitation system, extraction method, temperature and contact time (Migliato, 2011).

Then, the present study aims to optimize the conditions for obtention of standardized extracts from aerial parts of *L. origanoides*, as well as to investigate the hypotensive effect on blood pressure and the toxicological profile, focusing on its industrial application as a promising antihypertensive herbal drug.

2. Material and methods

2.1. Drugs and reagents

The 99.5% ethanol (Isofar, Brazil) and ultrapure water (Milli-Q®) were used for the extraction from raw materials and for analyzes. Naringenin was purchased from Sigma–Aldrich ($C_{15}H_{12}O_{15}$, 95.0%). Methanol (HPLC Grade, Synth, SP, Brazil) and acetic acid (CH_3COOH, HPLC Grade, Vetec, Brazil) were used in analyzes by Ultra-fast Liquid Chromatography (UFLC).

2.2. Botanical material

The aerial parts of *L. origanoides* H.B.K. were collected in José de Freitas, Piauí, Brazil, in 2013. The determination of the species was realized at the Herbarium Graziela Barroso from Federal University of Piauí. The voucher specimen was deposited under no. TEPB 9.205.

2.3. Optimization of the extraction process

The efficiency of the extraction process was evaluated by 2^3 factorial design where three parameters were analyzed, as follows: (1) solvent (ethanol or ethanol /water (1:1)); (2) sonication (present or absent); and (3) solvent exchange (daily during three days or every three days, totaling nine days). Eight different extraction systems were prepared in triplicate. The plant material (aerial parts) was previously dried at room temperature and triturated in a Wiley mill (Model MA 680 series and 98 in 2422, Marconi's, Brazil). Thereafter, 5 g of powder was added to 40 mL of solvent. For sonication, a Cleanner Thornton T1425 ultrasound device (Marconi's, Brazil) was used.

Samples of 10 mL of extracts obtained from each system were separately transferred to identified and weighed flasks, and then subjected to drying. Afterwards, the flasks were kept at 105 °C for 3 h. After drying, the samples were cooled in a desiccator and then weighed. The weighing was repeated after 30 min to check for consistency in the results. The masses of the residues obtained were used as a parameter for analysis and expressed in mg mL^{-1}.

2.4. Preparation of standardized hydroalcoholic extract (Lo-HAE)

The extraction conditions which provided the highest yields in solid residue were replicated in order to obtain standardized extracts to be used in future analysis. The aerial parts from *L. origanoides* were submitted to maceration in ethanol/water (1:1) for three days, followed by filtration and daily renewal of the solvent. The *L. origanoides* hydroalcoholic extract (Lo-HAE) was submitted to Laborota 400 rotary evaporator (Heidolph, Germany) coupled to a Kohlbach vacuum pump for recovery of ethanol (60 °C, cooling to 11 °C under a pressure of 600 mmHg). The Lo-HAE was dried in a lyophilizer coupled to a high vacuum pump (Edwards Micromodulyo freeze dryer/ValPump Savant VLP80).

2.5. Identification and quantification of Naringenin in Lo-HAE by UFLC

For the best wavelength for analysis of naringenin content in Lo-HAE, UV scanning was conducted for Lo-HAE (0.12 mg mL^{-1}) and naringenin (0.03 mg mL^{-1}). These assays were made in ethanolic solutions at the wavelengths range of 200–800 nm by using a double beam spectrophotometer (Lambda 25 UV/vis; PerkinElmer, MA, USA). After detection of the maximum absorption wavelength at 290 nm, Lo-HAE and naringenin were submitted to Ultra-Fast Liquid Chromatography (UFLC) (Shimadzu Prominence, Japan), provided with DGU-$20A_3$ degasser and a binary pump Shimadzu LC-20AD coupled to a UV-PDA detector. The column C18 Shimpack XR-ODS (2 mm × 30 mm) was used, and the mobile phase consisted of a mixture of methanol:acidified water (0.1% CH_3COOH) at a flow rate of 0.25 mL min^{-1}, with an increasing methanol gradient, as follows: 2% methanol at 0–2 min; 10% methanol at 5 min; 20% methanol at 7 min and 100% methanol at 30–35 min. The detection wavelength was selected at 290 nm and the injection volume was 5.0 μl.

Before the chromatographic analyses by UFLC, the Lo-HAE was submitted to a clean-up by a solid phase extraction (SPE) cartridge (Chromabond®, C18ec). The cartridge was activated with MeOH, Lo-HAE (30 mg) was solubilized in 500 μL of mobile phase, and subsequently submitted to SPE with methanol. After exhaustive extraction, the methanol eluate was filtered (nylon filter, 0.2 μm, Titan3) and then dried in a rotary evaporator.

For the determination of naringenin content in Lo-HAE, Lo-HAE after the clean-up procedure (6.6 mg mL^{-1}/MeOH) or naringenin (0.5 mg mL^{-1}/MeOH) were injected into UFLC. In another set of experiments, the co-injection of Lo-HAE and naringenin (1:1) or Lo-HAE and the equal volume of methanol were performed. An analytical curve from six different concentrations of naringenin (1.56, 2.08, 3.12, 3.64, 4.16 and 4.68 mg L^{-1}) and Lo-HAE at concentration of 49.5 mg L^{-1} were prepared and analyzed by UFLC in triplicate in order to determine naringenin content in Lo-HAE. The signal area related to the naringenin in the Lo-HAE solutions was calculated and the straight-line equation was determined.

2.6. Evaluation of Lo-HAE-induced hypotensive effect in rats

2.6.1. Animals

Female Wistar rats (230 ± 30 g, 3 months-old) were kept in cages and separated by experimental group. The temperature of the animal houses was maintained at 24 ± 2 °C under light–dark cycles of 12/12 h. Water and food were given ad libitum. After experiments, euthanasia was performed by intraperitoneally administration of sodium thiopental (100 mg/kg). This procedure is in accordance with Resolution no. 1000/2012 from the Federal Council of Veterinary Medicine from Brazil. This study was approved by the Ethics

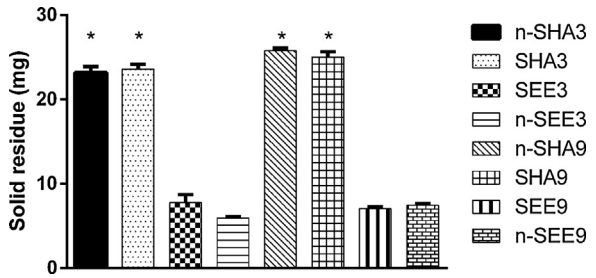

Fig. 1. Optimization of *Lippia origanoides* H.B.K. extraction by factorial design: n-SHA3 and n-SHA9: non-sonicated hydroalcoholic extract with 3 or 9 days extraction; SHA3 and SHA9—sonicated hydroalcoholic extract with 3 or 9 days extraction; SEE3 and SEE9: sonicated ethanolic extract with 3 or 9 days extraction, n-SEE3 and n-SEE9: non-sonicated ethanolic extract with 3 or 9 days extraction). ANOVA one-way followed by Tukey post-test. *$p < 0.0001$ vs SEE3, n-SEE3, SEE9 and n-SEE9.

Committee for Animal Experimentation of the Federal University of Piauí (no. 008/12).

2.7. Hypotensive effect after intravenous administration of Lo-HAE

This procedure was performed according to Mendes et al. (2014). Briefly, the aorta artery and cava vein from female Wistar rats ($n = 5$) were accessed by cannulation of the femoral artery and vein, respectively. Polyethylene PE-10 catheters connected to PE-50 catheters were used. The arterial catheter was connected to a pre-calibrated pressure transducer (Statham P23 ID, Gould, Cleveland, OH, EUA) coupled to a data acquisition system (AECAD 04H, AQCAD 2.3.9, AVS Projetos, SP, Brazil). The vein catheter was used for drug administrations. Sodium nitroprusside (10 mg/kg, i.v.) was administrated as positive control in order to verify the correct catheter implantation. After recovering from anesthesia (24 h), the pulse pressure (PP) and the heart rate (HR) was measured, and then the mean arterial pressure (MAP) was calculated. Then, Lo-HAE was intravenously administered at doses of 12.5, 25 and 50 mg/kg and at, and MAP and HR values were obtained.

2.7.1. Hypotensive effect after oral administration of Lo-HAE

The systolic blood pressure (SBP) was measured using the tail plethysmometer ML125 NIBP (AD Instruments, Australia), which consists of a tail cuff coupled to a tail pressure transducer which is connected to a data acquisition system (PowerLab, LabChart® 6.0, AD Instruments, Sydney, Australia). The procedures were performed according to El-Mosallamy et al. (2012) with modifications. Female Wistar rats were previously adapted to the restraint conditions during two weeks, in order to reduce the influence of stress induced by manipulation of the animal. Afterwards, the animals were previously conditioned at 35 ± 2 °C during 15 min and then they were treated with saline ($n = 6$) or Lo-HAE at doses of 100 mg/kg ($n = 6$) or 200 mg/kg ($n = 6$). Thereafter, they were placed in the restraint device and after tail cuff inflation/deflation, the SBP values (mmHg) were obtained for each animal after three sequential measurements, from 0 to 390 min and at intervals of 30 min.

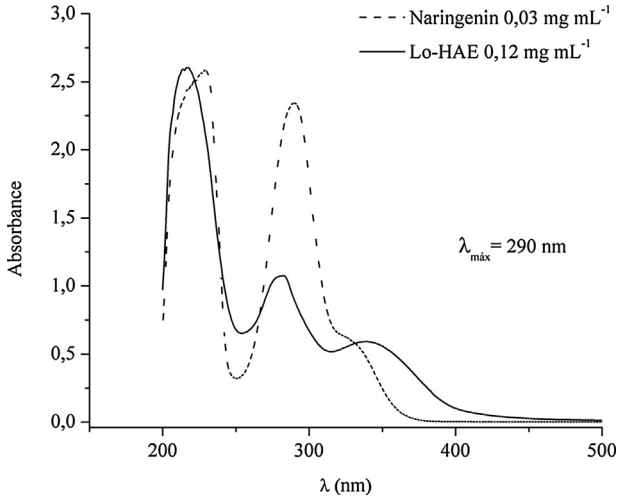

Fig. 2. Scan spectrum from hydroalcoholic extract of *Lippia origanoides* (Lo-HAE) and solution naringenin.

2.8. Acute oral toxicological evaluation

The toxicological evaluation of Lo-HAE was assessed in accordance to the Acute Toxic Class Method from the Organization for Economic Cooperation and Development (OECD), guide No. 423 (OECD, 2002a). In this experiment, female Wistar rats were firstly divided in Control and Treated groups ($n = 3$/group). Control group was orally treated with distillate water (1 mL/100 g, b.w.). In treated group, Lo-HAE was orally administrated at the single dose of 2000 mg/kg (1 mL/100 g, b.w.).

Afterwards, the animals were weighed daily and clinical and behavioral parameters were evaluated according to the OECD Guidance Document No. 19 (OECD, 2002b) and monitored during the first hour, and then after 2 h, 3 h, 4 h, 8 h, 24 h and each 24 h for 14 days. The side effects were classified as follows, when applied: (0) no effect, (−) decreased effect, (+) increased effect, (++) intense effect and (X) death.

After the first 72 h of observation, the test was repeated as indicated above with other groups of 3 animals each when the death of 0 or 1 animal per group had occurred, according to OECD No. 423 (OECD, 2002a). On the 14th day, the animals were euthanized and the internal organs (heart, liver, lung, spleen and kidneys) were removed and submitted to a gross pathological evaluation (color, texture and consistency). Then, the relative weights of organs were obtained as follows: relative organ weight = [organ weight (g)] × 100/[animal weight on the day of necropsy (g)].

2.9. Statistical analysis

The optimization of the extraction method was analyzed by ANOVA with Tukey's post-test. The results of hypotensive effect were expressed by the difference between MAP, SBP or HR values measured before and after treatments. The results obtained for direct measurement of MAP were evaluated by unpaired Student *t* test. For statistical significance of SBP measurements by tail plethysmography and evaluation of weight gain in the toxicological assessment, the Two-way ANOVA followed by Bonferroni's post-test were performed. In all analyzes we used the GraphPad® Prism 6.0 software to plot the graphs. The results were expressed as mean SEM, and statistical significance was considered when $p < 0.05$.

3. Results and discussion

3.1. Optimization of the extraction process from L. origanoides aerial parts

The standardization of herbal drugs can be defined as a set of actions to ensure the chemical quality, the therapeutic effect and the amount of active compounds of a product based on medicinal plants and can not be considered scientifically valid if active chemicals have not been validated and characterized by means of analytical and bioanalytical tests, in order to ensure the reproducibility in the production (Chouhdhary and Sekhon 2011). Minimizing these variables of the raw material of medicines

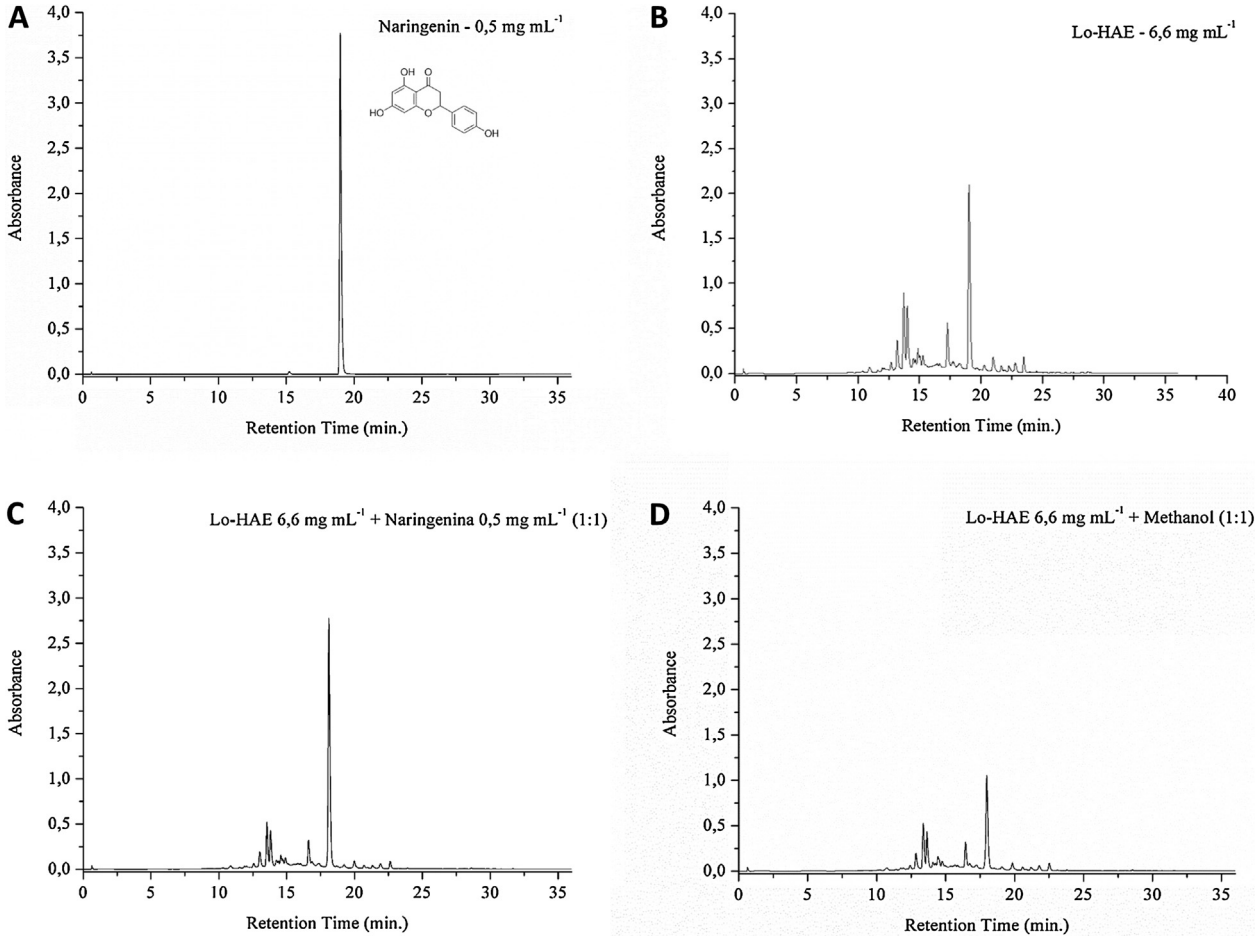

Fig. 3. UFLC chromatogram obtained from: Naringenin standard (0.5 mg mL^{-1}) (A) and Lo-HAE (6.6 mg mL^{-1}) (B) in methanol solution; co-injection of Lo-HAE (6.6 mg mL^{-1})/standard (0.5 mg mL^{-1}) (1:1) (C) and Lo-HAE (6.6 mg mL^{-1})/methanol(1:1) (D).

based on plant is the biggest challenge in the production of herbal medicines, which is a fundamental requirement to consolidate the parameters for quality, efficacy and safety.

The statistical analysis of the dry residue values obtained from 8 different extraction systems has demonstrated the highest yields when the extractions were performed by hydroalcoholic solutions, independently of sonication and time of extraction (Fig. 1). According to Migliato et al. (2011), extractive processes markedly depend on the diffusion phenomena. The renewal of the solvent associated with the agitation of the system can also determine the dissolution velocity and duration of the extraction process. Otherwise, some components from the sample could be exhausted in the course of extraction during the preparation of plant extracts, which indicates the yield of some processes demonstrates to be independent of time of extraction. Likewise, Salvalaggio et al. (2015) has demonstrated that the extraction time has no influence in the performance of extraction in various plant species.

In this sense, *L. origanoides* seems to have a particular response to the extractive process, due to the absence of significant differences in the efficiency of the extractive process associated to a prolonged contact time with the solvent or the occurrence of sonication. Furthermore, the highest yields were observed in hydroalcoholic rather than alcoholic extraction systems which reinforce the potential for lower cost applications of *L. origanoides* in obtention of standardized plant extracts. Therefore, this positive characteristic of this species is highlighted, probably leading efforts towards the applicability of *L. origanoides* in large-scale production of medicaments, regarding the obtention of extracts with assured quality, less time and low-cost industrial process.

3.2. Determination of naringenin content in Lo-HAE

The UV-scanning spectrum of Lo-HAE has shown a pronounced absorption band between 280 and 290 nm, while naringenin has maximum absorbance at 290 nm (Fig. 2). According to Agrawal (1989), phenol absorptions in the ultraviolet region (UV) display two absorption bands characteristic of flavonoids. Band II (240–285 nm) is related to ring A, while band I (300–550 nm) indicates absorption of ring B. Specifically, the group of flavanones, such as naringenin, shows the absorption of band II at the region of 275–295 nm and band I at the region of 300–330 nm. Thus, the spectrophotometric scanning confirms the presence of flavonoids in Lo-HAE as well as indicates the presence of naringenin in Lo-HAE, since the spectrum region of maximal absorption of naringenin coincides with the respective region of Lo-HAE.

The presence of naringenin in Lo-HAE could be confirmed by their respective UFLC chromatograms, where both major signals occur at the retention time of 18 min (Fig. 3A and B). Then, in order to confirm the identity of naringenin in Lo-HAE, the chromatogram of co-injection was obtained. Interestingly, no new signal has shown (Fig. 3C). These results confirm the identity of the major signal as naringenin. Furthermore, after comparison between co-injection chromatograms of Lo-HAE/naringenin (1:1) and Lo-HAE/MeOH (1:1), both major signals were observed at retention time of 18 min. The major signal of co-injection chro-

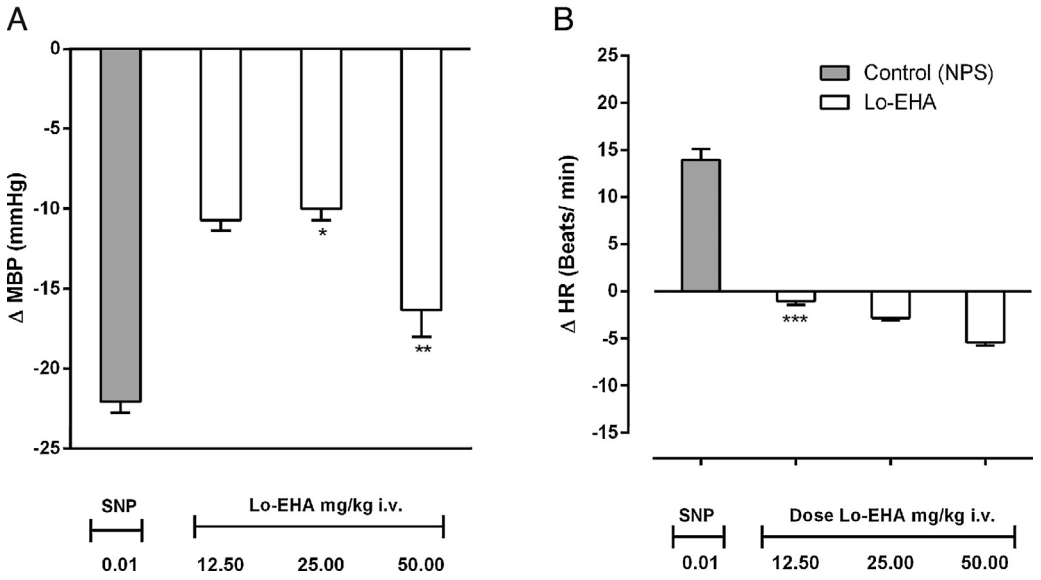

Fig. 4. Effects of the standardized extract from *L. origanoides* on mean arterial pressure (A) and heart rate (B) after intravenous administration. Values are expressed as mean ± SEM. *t* Student test unpaired. $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$ vs control group.

matogram of Lo-HAE/naringenin (1:1) increases approximately 1.8-fold when compared with the respective signal in the co-injection chromatogram of Lo-HAE/MeOH (1:1), while other peaks showed similar intensity (Fig. 3D). These findings reinforce the identity of naringenin as the major compound of Lo-HAE.

For quantification of naringenin in the Lo-HAE, the area of the major signal in the UFLC chromatogram of the Lo-HAE (49.5 mg L^{-1}) was calculated. Considering the linearity observed in the analytical curve which expresses the concentration of naringenin related to the intensity of signal area ($R^2 = 0.99866$), data obtained for signal area were plotted in the following line equation: $y = 42.313$, 5809×-11226, 3496, and naringenin content was determinated in Lo-HAE at concentration of 37.3 mg g^{-1} (3.73 g%) of Lo-HAE. In a previous study, the naringenin content in methanol extracts from different chemotypes of *L. origanoides* has been determinated, and the highest naringenin content was 5.67 mg g^{-1} of plant material (Stashenko et al., 2013), while in the present work, a naringenin content of 10.92 mg g^{-1} of plant material was observed. Hence, the higher naringenin content determinated in Lo-HAE reinforces this compound as a promising biomarker of *L. origanoides*.

Therefore, from the results desmostrated we can glimpse the potential industrial use of products derived from the plant species *L. origanoides*, since the standard and optimized extraction methods allow advantages such as shortening of the extraction time, reduced use of solvents, increasing the extraction yield and the possibility of controlling the quality of the extracts produced through the use of measurable biomarkers.

3.3. The Lo-HAE induces hypotensive effect in rats

The intravenous administration of Lo-HAE promoted hypotensive effect by decrease of mean arterial pressure at the doses of 12.5; 25 and 50 mg/kg (ΔMAP in mmHg: -10.72 ± 0.65, -10.01 ± 0.71 and -16.34 ± 1.69, respectively) followed by a slight bradycardic effect (HR in bpm: -1.03 ± 0.40, 0.26 ± -2.81 and -5.43 ± 0.30, respectively) (Fig. 4A and B). Besides, the oral administration of Lo-HAE it also promoted a marked decrease of systolic blood pressure (SBP) (Fig. 5) at the doses of 100 and 200 mg/kg. A progressive time-dependent decrease of SBP was observed at the dose of 200 mg/kg with maximal reduction 120 min after administration and duration of 300 min (Control: 1.41 ± 2.45 mmHg; Lo-HAE: -19.09 ± 2.54 mmHg). Likewise, Lo-HAE at dose of 100 mg/kg promotes maximal hypotensive effect after 60 min of administration of Lo-HAE followed by an increase of SBP at 90 min and return to the maximal response at 120 min, with duration of 270 min (Control: 6.11 ± 1.19 mmHg; Lo-HAE: -19.12 ± 2.95 mmHg).

The duration of the Lo-HAE-induced hypotensive effect after oral administration was approximately 5.0 h. Krepsky (2011) has reported the short-term reduction for 2 h of approximately 20 mmHg in systolic blood pressure of normotensive rats after oral administration of *Cuphea carthagenensi*. The duration of this action is quite short, and then its potential application in hypertensive urgencies or emergencies seems to me more applicable. Hence, the long-term effect such as observed after oral administration of Lo-HAE represents an advantage in the development of herbal medicines for chronic treatment of hypertension due to the adequate posology in accordance with the appropriate design of the formulation.

Although mechanisms underlying Lo-HAE-induced hypotensive effect have not been elucidated, several studies have reported the narigenin-induced vasodilator effect and the underlying mechanisms, such as the involvement of activation of large conductance calcium-activated potassium channels (BK_{Ca}^{2+}) (Saponara et al., 2006), as well as the increase in cytosolic cAMP and cGMP probably mediated by inhibition of PDE isoforms (Orallo et al., 2005). Furthermore, Mladěnka et al. (2010) has demonstrated that the presence of 4-keto group in flavonoid structure is a prerequisite for the vasodilation, and this feature is found in the molecular structure of the four main flavonoids present in *L. origanoides* (naringenin, pinocembrine, quercetin and luteolin).

Furthermore, naringenin has demonstrated an antiatherogenic potential, and good results have been shown in cardioprotective studies (Chang et al., 2010; Lee et al., 2012; Testai et al., 2013), as well as in functional studies on vascular function of streptozotocin-induced diabetic rats (Fallahi et al., 2012). Therefore, considering the high concentration of flavones of *L. origanoides*, it is crucial to investigate the cardiovascular effects of this species, these evidences may probably be related with the hypotensive effect observed for Lo-HAE, and the development of herbal medicines from *L. origanoides* is then reinforced.

Furthermore, the search for new therapeutic alternatives for arterial hypertension treatment is relevant due the high number of

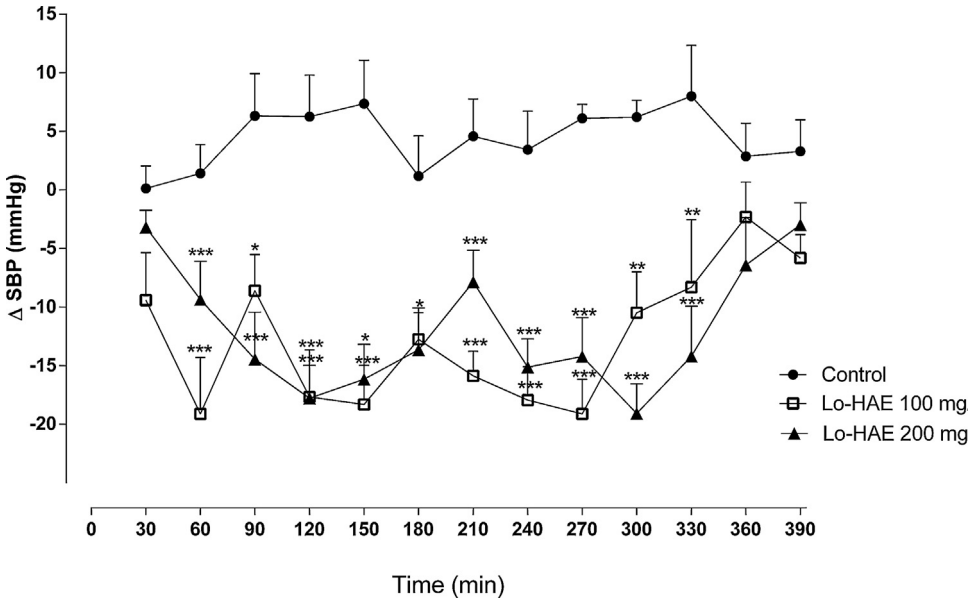

Fig. 5. Variation in systolic blood pressure after oral administration of Lo-HAE in doses of 100 mg/kg and 200 mg/kg in normotensive rats. Values are expressed as mean ± SEM. Two-way ANOVA followed by Bonferroni post test. $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$ vs to the control group.

patients with uncontrolled hypertension, even in drug treatment, and many collateral effects by continuous use of drugs commonly used for hypertension (Bunte et al., 2013; Godinho, 2011). As part of this search, the bio-based material obtained from *L. origanoides*, showed to be promising, because products come from vegetal extracts which can be safer, less costly and low collateral effects even in higher doses (Calixto, 2000).

3.4. Acute oral toxicological evaluation of Lo-HAE

The use of herbal extracts from raw materials in order to produce medicines requires toxicological studies. The internationally validated tests which assess the acute oral toxicological of herbal extracts and chemicals are based on classifications according to their potential of toxicity or lethality, and they are in accordance with the Globally Harmonised System (GHS). An important example is the guidelines of the Organization for Economic Cooperation and Development (OECD). They are accepted by worldwide regulatory agencies, and therefore they are adopted for this study (Jonsson et al., 2013).

After acute oral treatment of animals with Lo-HAE at single dose of 2000 mg/kg, no deaths were observed. According to OECD no. 423, the protocol was repeated with a new group of 3 animals. The second set of treatment did not promote any deaths. The product-test is considered and 5000 mg/kg is encountered. Then, the estimated DL_{50} for Lo-HAE is higher than 2000 mg/kg, and therefore classified in the category X of GHS, which refers to non-classified substances (OECD, 2002a).

Besides, the acute oral administration of Lo-HAE did not promote any change of behaviour during the hippocratic screening, and there were no clinical signs of toxicity, when compared with the control group. Similarly, the administration of Lo-HAE had no significantly alteration to the body mass of the animals compared with the control group (Fig. 6). Additionally, some organs (liver, kidneys, heart, spleen and lungs) were evaluated macroscopically and no changes were observed in their size, rigidity, structure or color.

It must be considered that the observed results represent a preliminary assessment of the acute oral toxicological profile of Lo-HAE which provides information about the risks resulted from only a single acute exposure. Interestingly, the lack of acute oral toxicity of Lo-HAE leads to a promising result, and repeated dose oral toxicity studies as well as the development of Lo-HAE-based pharmaceutical formulations for the treatment of hypertension may be considered.

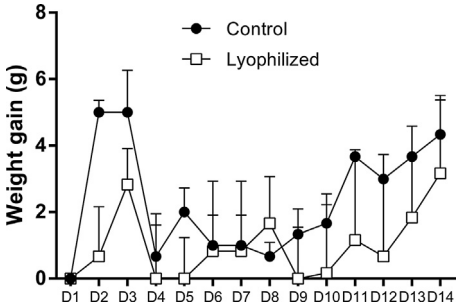

Fig. 6. Effects of the standardized extract from *L. origanoides* on weight gain in *Wistar* rats for 14 days. Two-way ANOVA followed by Bonferroni post test.

Thus, optimization and standardization of the methodology developed for obtaining bio-based material showed high technological potential due to its reproducibility for industrial scale production and the control produced by the biomarker identified material. In addition, this study is a pioneer in the evidence of hypotensive activity of the *L. origanoides*, thus developing great expectation on future production of new therapeutic alternatives for the treatment of hypertension.

4. Conclusions

An efficient standardized hydroalcoholic extraction process was obtained from aerial parts of *L. origanoides*, and the presence of naringenin as the major compound reinforces its role as a potential biomarker for the Lo-HAE assessment. Additionally, the administration of Lo-HAE to female Wistar rats promoted pronounced hypotension by both intravenous and oral routes. In addition, no sign of toxicity after single oral dose at 2000 mg/kg was observed. These findings lead the Lo-HAE towards the elucidation of mechanism underlying Lo-HAE-induced cardiovascular effects, as well as

the development of herbal medicines for application in treatment of arterial hypertension.

Acknowledgements

The authors are grateful to UFPI(Universidade Federal do Piauí), CNPq (Conselho Nacional para Desenvolvimento Científico e Tecnológico) and FAPEPI (Fundação de Amparo à Pesquisa do Estado do Piauí, Brazil) for the financial support, as well as to Mr. Luis Mário Rezende Júnior for the assistance with English proofreading. This work was granted by FAPEPI/SESAPI/MS/CNPq: "Programa Pesquisa para o SUS—PPSUS", grant number EFP_00007002.

References

Agrawal, P.K., 1989. Carbon-13 NMR of Flavonoids. v. 39. Elsevier Science Publishing Company Inc., Amsterdam.

Barreto, H.M., Fontinele, F.C., Oliveira, A.P., Arcanjo, D.D.R., Santos, B.H.C., Abreu, A.P.L., Coutinho, H.D.M., Silva, R.A.C., Sousa, T.O., Medeiros, M.G.F., Citó, A.M.G.L., Lopes, J.A.D., 2014. Phytochemical prospection and modulation of antibiotic activity in vitro by lippia origanoides H.B.K. in methicillin resistant *Staphylococcus aureus*. Biomed. Res. Int. 2014, 1–7, http://dx.doi.org/10.1155/2014/305610.

Brazil, 2006. MINISTÉRIO DA SAÚDE Decreto n°. 5.813 de 22 de junho de 2006. Política nacional de plantas medicinais e fitoterápicos. Disponível em: http://portal.saude.gov.br/portal/arquivos/pdf/decreto_5813_fito.pdf (accessed 29.09.15.).

Bunte, M.C., Oliveira, E.I., Shishehbor, M.H., 2013. Endovascular treatment of resistant and uncontrolled hypertension. J. Am. Colloid Cardiol. Interventions 6, 1–9, http://dx.doi.org/10.1016/j.jcin.2012.09.005.

Calixto, L.B., 2000. Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents). Braz. J. Med. Biol. Res. 33, 179–189.

Chang, C., Wang, G., Zhang, L., Tsai, W., Chen, R., Wu, Y., Kuo, Y., 2010. Cardiovascular protective flavonolignans and flavonoids from *Calamus quiquesetinervius*. Phytochemistry 71, 271–279, http://dx.doi.org/10.1016/j.phytochem.2009.09.025.

Chouhdhary, N., Sekhon, B.S., 2011. An overview of advances in the standardization of herbal drugs. J. Pharm. Educ. Res. 2, 55–70.

El-Mosallamy, A.E.M.K., Sleem, A.A., Abdel-Salam, O.M.E., Shaffie, N., Kenawy, S.A., 2012. Antihypertensive and cardioprotective effects of pumpkin seed oil. J. Med. Food 15, 180–189, http://dx.doi.org/10.1089/jmf.2010.0299.

Fallahi, F., Roghani, M., Moghadami, S., 2012. Citrus flavonoid naringenin improves aortic reactivity in streptozotocin-diabetic rats. Indian J. Pharmacol. 44, 382–386, http://dx.doi.org/10.4103/0253-7613.96350.

Guerrero, M.F., Puebla, P., Carrón, R., Martín, M.L., Arteaga, L., San Román, L., 2002. Assessment of the antihypertensive and vasodilator effects of ethanolic extract of some Colombian medicinal plants. J. Ethnopharmacol. 80, 37–42, http://dx.doi.org/10.1016/s0378-8741(01)00420-2.

Jonsson, M., Jestoi, M., Nathanail, A.V., Kokkonen, U., Anttila, M., Koivisto, P., Karhunen, P., Peltonen, K., 2013. Application of OECD Guideline 423 in assessing the acute oral toxicity of moniliformin. Food Chem. Toxicol. 53, 27–32, http://dx.doi.org/10.1016/j.fct.2012.11.023.

Krepsky, P.B., 2011. Composição química quantitativa e avaliação da potencial atividade vasodilatadora de Cuphea carthagenensis (Jacq.). In: MacBride. Tese (Doutorado em Ciências Farmacêuticas). Universidade Federal de Minas Gerais, Belo Horizonte.

Lee, J., Yi, H., Kim, I., Kim, Y., Nhiem, N.X., Kim, H.O., Myung, C., 2012. (2S)-Naringenin from *Typha angustata* inhibits vascular smooth muscle cell proliferation via a G0/G1 arrest. J. Ethnopharmacol. 139, 873–878, http://dx.doi.org/10.1016/j.jep.2011.12.038.

Mendes, M.B., Silva-Filho, J.C., Sabino, C.K.B., Arcanjo, D.D.R., Sousa, C.M.M., Costa, I.C.G., Chaves, M.H., Oliveira, R.C.M., Oliveira, A.P., 2014. Pharmacological evidence of α_2-adrenergic receptors in the hypotensive effect of *Platonia insignis* Mart. J. Med. Food 17, 1079–1085, http://dx.doi.org/10.1089/jmf.2013.0151.

Mladěnka, P., Zatloukalová, L., Filipský, T., Hrdina, R., 2010. Cardiovascular effects of flavonoids are not caused only by direct antioxidant activity. Free Radic. Biol. Med. 49, 963–975, http://dx.doi.org/10.1038/sj.bjp.0706951.

Migliato, K.F., Corrêa, M.A., Salgado, H.R.N., Tognolli, J.O., Sacramento, L.V.S., Mello, J.C.P., Giannini, M.J.S.M., Almeida, F.A.M., Pizzolitto, A.C., 2011. Planejamento experimental na otimização da extração dos frutos de *Syzygium cumini* (L.) Skeels. Quim Nova 34, 695–699, http://dx.doi.org/10.1590/s0100-40422011000400024.

OECD, 2002a. Test No. 423: acute oral toxicity—acute toxic class method. In: OECD Guidelines for the Testing of Chemicals, Section 4. OECD Publishing, Paris, http://dx.doi.org/10.1787/9789264071001-en.

OECD, 2002b. Guidance document on the recognition, assessment and use of clinical signs as human endpoints for experimental animals used in safety evaluation. In: OECD Series on Testing and Assessment, No. 19. OECD Publishing, Paris, http://dx.doi.org/10.1787/9789264078376-en.

Orallo, F., Camiña, M., Álvarez, E., Basaran, H., Lugnier, C., 2005. Implication of the cyclic nucleotide phosphodiesterase inhibition in the vasorelaxant activity of the citrus-fruits flavonoid (±)-naringenin. Planta Med. 71, 99–107, http://dx.doi.org/10.1055/s-2005-837774.

Pascual, M.E., Slowing, K., Carretero, E., Sánchez Mata, D., Villar, A., 2001. Lippia: traditional uses, chemistry and pharmacology: a review. J. Ethnopharmacol. 76, 201–214, http://dx.doi.org/10.1016/S0378-8741(01)00234-3.

Saponara, S., Testai, L., Iozzi, D., Martinotti, E., Martelli, A., Chericoni, S., Sgaragli, G., Fusi, F., Calderone, V., 2006. (+/−)-Naringenin as large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel opener in vascular smooth muscle cells. Br. J. Pharmacol. 149, 1013–1021, http://dx.doi.org/10.1038/sj.bjp.0706951.

Salvalaggio, M.O., Freitas, R.A., Franquetto, E.M., Koop, H.S., Silveira, J.L.M., 2015. Influence of the extraction time on macromolecular parameters of galactomannans. Carbohydr. Polym. 116, 200–206 http://dx.doi.org/10.1016/j.carbpol.2014.05.036.