



Original Article

Development of an oral suspension containing dry extract of *Aleurites moluccanus* with anti-inflammatory activity



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ABSTRACT

Aleurites moluccanus L. (Willd.), Euphorbiaceae, is a tree that is native to Indonesia and India. Various parts of this tree are commonly used in traditional medicine to treat pain, fever, inflammation, hepatitis, gastric ulcer and other ailments. An oral suspension containing dried extract of *A. moluccanus* was developed and *in vivo* anti-inflammatory activity was evaluated. Extract 100 and 50 mg/ml loaded oral suspensions were prepared using different suspending agents. The formulations were analysed by their appearance, pH, density, redispersion time, rate of settling, rheological behaviour, distribution of particle size and zeta potential. The dose uniformity was determined by measuring the content of total phenolic compounds expressed in swertisin by a validated HPLC method, as well as the dissolution profile. The stability of oral suspensions was analysed in accelerated studies (40 °C for 6 months). The anti-inflammatory activity was analysed using an *in vivo* paw oedema model. The taste and odour of the suspensions were shown to be characteristic of the extract. Carmellose sodium (CS; 0.5%) and microcrystalline cellulose and carmellose sodium mixture (MCCS; 1%) showed better physical behaviour. The content of total phenolic compounds was 1.6 mg/ml and approximately 100% of the total phenolic compounds dissolved within 10 min. During the stability study, the formulations were approved by their physical–chemical properties and were shown to lose 12–14% of total phenolic compounds at 40 °C after 6 months. Suspensions containing 50 mg/ml of standardised dried extract inhibited around $35 \pm 7.6\%$ of paw oedema. Formulations containing CS showed more anti-inflammatory activity. Suspensions containing dry extract of *A. moluccanus* were successfully obtained and showed physical and physical–chemistry properties that were appropriate and characteristic of this dosage form, suitable for administration in paediatric and elderly populations, making this an alternative to tablets.

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Introduction

Aleurites moluccanus L. (Willd.), Euphorbiaceae, is a tree popularly known in Brazil as “nogueira-da-india” and as Kukui, Tuitui and Candlenut in other countries. This tree is originally from Indonesia and India and is widespread in tropical regions (Duke, 1991). Its use in traditional medicine is extensive. Various plant parts including seeds, leaves, flowers and bark are used in traditional medicine. There are reports of the use of the crushed seeds and toast for constipation, decoction of leaves in poultices for headache, fevers, ulcers, swollen joints, and gonorrhoea, the bark

is used for bloody diarrhoea and flowers and the sap of branches in the treatment of oral Candidiasis (Dunfors et al., 2002).

Phytochemical studies of leaves allowed isolation of the compounds swertisin (Meyre-Silva et al., 1997), the mixture of α and β -amyrin, η -hentriacontane, the mixture of stigmasterol, β -sitosterol and campesterol (Meyre-Silva et al., 1998) and 2''-O-ramnosil swertisin (Meyre-Silva et al., 1999). Furthermore, three minor flavonoids, derived from swertisin (Cesca et al., 2012a), and five megastigmanes (Silva et al., 2012), which might contribute to the potent anti-nociceptive effect of this species, were described.

The analgesic effect of the plant may be associated with the GABAergic and oxidonitrergic system and not be influenced by adrenergic, cholinergic, dopaminergic and opioid systems (Quintão et al., 2012). The *A. moluccanus* dry extract showed a potential anti-nociceptive effect in the inflammatory sensitisation model,

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using different inducing agents such as carrageenan, CFA or PGE₂, in mice. This biological effect can be attributed to the 2''-O-ramnosilswertisin and swertisin flavonoids, since they also showed activity in the tested models (Quintão et al., 2011). Semisolid preparations containing 0.5 and 1.0% of *A. moluccanus* leaves dry extract were developed and showed potential anti-inflammatory, antinociceptive and wound healing activities in *in vivo* pharmacological models (Cesca et al., 2012b).

The leaves dry extract was obtained by spray-drying in the laboratory and at an industrial scale, as this is a reproducible process and technological transformations do not change the pharmacological properties of the medicinal plant (Quintão et al., 2011). The dry extract was standardised using HPLC methodology employing swertisin and 2''-O-ramnosilswertisin as markers (Cesca et al., 2012a).

Further to the use of the semisolid formulation for topical application (Cesca et al., 2012b), coated tablets containing dry extract for oral use have also been developed in our research group. As the 250 and 500 mg dried extract tablets are relatively large due to the required load carrier, their use in children or elderly patients is restricted. Thus, the present study aimed to develop oral suspensions in which the *A. moluccanus* dried extract has a slightly soluble fraction but, during the spray drying process, includes the addition of an insoluble excipient (colloidal silicon dioxide), which prevents incorporation into a solution dosage form. Also, instead of quantifying only the two markers 2''-O-ramnosilswertisin and swertisin, in the present work, we quantified the total phenolic compounds of the extract, expressed in swertisin in view of measuring more components of the herbal matrix (besides the two mentioned markers, also the previously detected minor flavonoids and other unknown phenolic compounds). The HPLC methodology was successfully validated and applied for content uniformity and dissolution of the formulations. The suspensions were characterised and the *in vivo* anti-inflammatory activity was performed to ensure that the technological process did not change the pharmacological properties of the extract.

Material and methods

Material

Microcrystalline cellulose and carmellose sodium mixture (Avicel® RC 591) was purchased from FMC Biopolymer (Philadelphia, USA), carmellose sodium was purchased from DEG (São Paulo, Brazil); methanol and acetonitrile (HPLC grade) were obtained from J.T. Baker (Phillipsburg, New Jersey, USA), and water was purified using Easy Pure equipment (Waltham, Massachusetts, USA). Sorbitol and sucralose, pharmaceutical grade, were purchased from Via Farma (São Paulo, Brazil). The swertisin isolated from *A. moluccanus* leaves with a purity of >95% (Quintão et al., 2011) was used as a reference substance for the development and validation of the HPLC method. The other reagents were of analytical grade and used as received, without any further purification.

Table 1
Formulations of oral suspensions of *Aleurites moluccanus* dry extract.

Batches	I	II	III	IV	V	VI	VII	VIII	IX	X
Dry extract (mg/ml)	100	100	100	100	100	100	100	100	50	50
CS ^a (%)	1.0	1.0	0.5	0.5	–	–	–	–	0.5	–
MCCS ^a (%)	–	–	–	–	1.0	1.0	0.5	0.5	–	1.0
Sucralose (%)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Na benzoate (%)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Sorbitol (%)	–	50	–	50	–	50	–	50	20	20
Water	qsp	qsp	qsp	qsp	qsp	qsp	qsp	qsp	qsp	qsp

^a CS, carmellose sodium; MCCS, microcrystalline cellulose and carmellose sodium mixture.

Herbal material

A voucher specimen of *Aleurites moluccanus* L. (Willd.), Euphorbiaceae, collected in July 2007 in Tijucas (State of Santa Catarina, Brazil) and identified by Prof. Dr. Ademir Reis (Department of Botany/Santa Catarina Federal University, Florianópolis, Brazil), was deposited at the Barbosa Rodrigues Herbarium (Itajaí, Brazil), under number VC Filho 001.

Dry extract of *A. moluccanus* containing 2''-O-ramnosilswertisin and swertisin at concentrations of 3.0% and 0.4%, respectively (Cesca et al., 2012a), was prepared on an industrial scale (Centroflora, Botucatu, Brazil) from dry leaves. The extract was prepared by maceration with a 1:10 drug:solvent ratio and 7:3 ethanol:water as a solvent, for five days. After extraction, filtration and concentration, the extract was mixed with colloidal silicon dioxide and dried for the spray-drying method using inlet and outlet temperatures of 165–180 °C and 70–80 °C, respectively. This extract was previously characterised (Quintão et al., 2011).

Preparation of *A. moluccanus* suspensions

Suspensions containing 100 mg/ml and 50 mg/ml dry extract of *A. moluccanus* were prepared according to the formulations shown in Table 1. The dry extract used was prepared on an industrial scale and containing colloidal silicon dioxide (approx. 25%) as dry adjuvant. Initially, the suspensor vehicle was prepared using water or water/sorbitol as a vehicle, CS and MCCS as the suspending agent, sodium benzoate as the preservative and sucralose as a sweetener. The dry extract was wet and dispersed in the suspensor vehicle. The suspensions were stored in amber glass bottles for future analysis.

Analysis of the organoleptic characteristics and pH

In the organoleptic characteristics analysis, colour, physical aspects, odours and taste were observed by direct perception and/or sensory experimentation.

The pH value of the formulations was determined by direct reading in a potentiometer at 23 ± 2 °C, which was previously calibrated in pH 4 and 7. The analysis was performed in triplicate.

Analysis of settling behaviour

The suspensions were homogenised and transferred into a graduate cylinder and the volume of sediment was observed after 1, 2, 4, 6, 8 and 24 h. The rates of settling, *F*, which is defined as the ratio of the final settled volume *V_u* to the original volume *V_o*, was calculated using Eq. (1) (Florence and Attwood, 2006). The assay was performed in triplicate.

$$F = \frac{V_u}{V_o} \quad (1)$$

The redispersion time was determined by manual stirring according to Tagliari et al. (2009). Approximately 40 ml of suspension was stored in 60 ml glass bottles (approximately two-thirds

of the volume). After 24 h, the bottles were inverted to 180° and the necessary time for homogenisation of the disperse phase was observed.

Rheological analysis

The rheological behaviour was performed in a rotational viscometer (Haake VT550, Waltham, USA), at $25 \pm 0.1^\circ\text{C}$, using an SV DIN sensor with a shear rate increase of 6.5 to 100 s^{-1} in 150 s, a constant shear rate of 100 s^{-1} for 150 s and a shear rate decrease of 100 – 6.5 s^{-1} in 150 s. The rheological behaviour, viscosity average and thixotropy were assessed. The flow behaviour index of pseudo-plasticity was calculated using the Ostwald-de Waele model (Eq. (2)), where τ is the shear stress (Pa), γ is the shear rate (s^{-1}), k is the flow consistency index (Pa s^n) and n is the flow behaviour index. The analysis was performed in triplicate.

$$\tau = k \cdot \gamma^n \quad (2)$$

Assay and content uniformity analyses

For HPLC analysis, a Phenomenex® C8 column (150 mm \times 4.6 mm, 5 μm) was used, with acidified water (pH 3.5, acetic acid) (solvent A) and acetonitrile (solvent B) as the mobile phase in the gradient method from 90:10 (A:B, v/v) (0 min) to 75:25 (A:B, v/v) (27 min), to 90:10 (A:B, v/v) (30 min), and then maintaining the latter for 35 min, at a flow of 0.6 ml/min, 30°C , with detection at 254 and 338 nm.

The total phenolic assay (TP) expressed in swertisin was previously determined for the *A. moluccanus* reference dried extract, using the above HPLC methodology and the swertisin as standard. Briefly, the sum of the selected peaks in the reference extract chromatogram was expressed in swertisin concentrations, taking into account the area and potency of the swertisin standard. This standardised reference extract was further used as a reference solution to quantify these markers in the formulations, as described below.

The assay of the suspensions containing 50 mg/ml dry extract were determined by HPLC and calculated TP expressed in swertisin using equation 3, where AA and A_p are the sum of the integrated areas of the peaks corresponding to compounds which absorb at 254 nm (total phenolic) in the sample and reference solution, respectively; M_p is the weight (mg) of *A. moluccanus* dry extract in dry basis used to prepare the reference solution; Pot is the extract potency (%) of the reference solution previously determined against swertisin (3.21% TP) and DF is the dilution factor.

$$\text{TP (mg/ml)} = \frac{AA \cdot M_p \cdot Pot}{A_p \cdot 100} \times DF \quad (3)$$

After stirring for 10 min, 10 ml of suspension was transferred to a 250 ml volumetric flask using methanol and acidified water (pH 3.5, acetic acid) 1:1 v/v as solvent, which was sonicated for 30 min, resulting in the sample solution at a concentration of 2 mg/ml. The solution was filtered through a 0.45 μm regenerated cellulose membrane filter and analysed by HPLC. The reference solution of dry extract, also at 2 mg/ml, was prepared using the procedure described for the sample solution. At least six individual injections of reference solution were performed to assess suitability, including resolution between peaks ($R > 1.0$) and the sum of area repeatability ($\text{RSD} < 2.0\%$).

For content uniformity determination, the suspensions were maintained for 24 h in the stagnancy state, stirring for 10 s, followed by sampling at 1 cm below the meniscus; these were then prepared according to the method described for the total phenolic assay. The assay was performed in ten different samples (Farmacopeia Brasileira, 2010).

Dissolution profile

The dissolution method was developed using different media (0.1 M HCl, acetate buffer solution at pH 4.5 and PBS buffer solution at pH 6.8) and stirring speeds (50, 75 and 100 rpm), using paddle apparatus; the method was performed at 37°C with a volume of 900 ml, according to the British Pharmacopoeia (2008), with small changes. The suspensions were stirred for 10 min and 10 ml of sample was collected using a syringe. The samples were filtered through paper and a 0.45 μm membrane and quantified by HPLC using 0.55 mg/ml of extract solution as a reference.

Stability study of suspensions

The stability study was performed in triplicate with 50 mg/ml suspensions using accelerated conditions (40°C), according to Anvisa (2005). The organoleptic characteristics, viscosity, redispersion time, rate of settling, pH and TP content were evaluated at time zero, 30, 90 and 180 days. Additionally, microbiological analyses (Farmacopeia Brasileira, 2010), potential zeta (Zetasizer, Malvern) and dissolution tests were performed at zero and 180 days.

Validation of analytical methods

The assay and dissolution methods were submitted to analytical validation (ICH, 2005). For the validation of both methods (assay and dissolution), the 50 mg/ml suspension was used. The selectivity of the method was realised by comparing chromatograms of the sample, the solvent, the reference extract and the mobile phase to detect any possible interference of the excipients or solvents. The resolution of the peaks (swertisin and 2''-O-ramnosil swertisin) was also calculated.

For assay method validation, the linearity was assessed by analysis of TP content in an analytical curve elaborated with a standard extract solution after a series of dilutions in the range of 0.5–5.0 mg/ml (with 2.0 mg/ml being the target concentration). Analysis of the analytical curve was also repeated in the presence of the suspension following the addition of 100 μl in each level of standard extract solution (spiked curve). The solutions were prepared in triplicate, injected twice and monitored at 254 nm. The relative standard deviations (RSD%) of the slope, intercept and the correlation coefficient (r) of the curves were calculated using Excel 5.0 software. The quantification limit (LOQ) was determined from the analytical curve (ICH, 2005).

The accuracy was determined by repeating the assay described in Dissolution profile section, adding known concentrations of standard extract solution in volumetric flasks with a fixed volume of suspension (5 ml); final concentrations were obtained for three levels (low, medium and high), in triplicate. The recovery (%) was calculated in each level, after discounting the assay of a sample solution (5 ml) without spiking.

The repeatability was determined by repeating the assay described in Dissolution profile section, six times on the same day by the same analyst. For intermediate precision, the repeatability was performed on two additional days by two different analysts. The average and RSD% of TP content were calculated (ICH, 2005).

The robustness was realised by changing the temperature (29, 30 and 31°C) and flow (0.5, 0.6 and 0.7 ml/min). The data were evaluated using a single factor analysis of variance (ANOVA) ($p < 0.05$).

The dissolution test was validated following the methodology described in the United States Pharmacopoeia (USP, 2012), by selectivity, linearity, precision and accuracy analysis. The selectivity was realised by comparing chromatograms of the sample, the solvent (dissolution medium), the standard extract, the mobile phase and placebo (suspension vehicle without extract) to detect any possible interference of the excipients or solvents. The target concentration

of 0.55 mg/ml was chosen and the method linearity was verified in the 0.2–0.8 mg/ml of concentration range. The accuracy was determined on three levels of standard addition, totalling final levels of 0.432 mg/ml, 0.556 mg/ml and 0.679 mg/ml, in triplicate. In each cube, 5 ml of suspension was added. The recovery (%) was calculated for each level, after discounting the average TP content dissolved in the cube without spiking. The precision of the method was evaluated by calculating the average and RSD% in the repeatability and intermediate precision. The repeatability was determined by repeating the dissolution test six times on the same day by the same analyst. For intermediate precision, the repeatability was repeated on one additional day.

Pharmacological study

Animals

Male Swiss mice (25–35 g, ten weeks of age; $n = 6$ per group), obtained from the University of Vale do Itajaí (Itajaí, Brazil), were used in this study. The animals were maintained under conditions of optimum temperature, light and humidity ($22 \pm 1^\circ\text{C}$, 12 h light/dark cycle, 60–80% humidity) with water and food provided *ad libitum*. All procedures used in this study followed recommendations of the *Principles of Laboratory Animal Care* guide from National Institutes of Health (NIH) publication no. 85-23, and were approved by the Univali Animal Ethics Committee (Protocol numbers 416/2008). Every effort was made to minimise animal suffering and reduce the number of animals used.

Carrageenan-induced paw oedema

The experiments were conducted in accordance to Tratsk et al. (1997) with some modifications. The animals received injections containing 50 μl of Carrageenan (0.3 mg/paw) under the surface of the right hind-paw. The other hind-paw received NaCl solution and was used as a control. The mice were pre-treated with the formulations (250 mg/kg, *p.o.*) or with the suspensor vehicle 1 h before the carrageenan injection. Indomethacin (10 mg/kg, *p.o.*) was used as a positive control. The oedema induced by carrageenan was evaluated 0.5, 1, 2, 4, 6 and 24 h after the injection and expressed in μl comparing the differences between the right and left hind-paws.

Statistical analyses

The results were expressed as mean \pm standard error of the mean (E.P.M. 95%). The percentage of the inhibition was shown using mean \pm standard error of the average of the difference (in percentage) between the areas under the curve (AUC) in relation to the control group. The statistical analyses of the data obtained were expressed using two-way analysis of variance (ANOVA) followed

by Bonferroni test, using one-way ANOVA followed by Dunnett or Newman-Keuls, or using the Student *T* test when suitable.

Results and discussion

To develop a new anti-inflammatory and analgesic herbal medicine from the dry extract of *A. moluccanus* leaves, tablets containing dry extract (250 and 500 mg) for oral administration (unpublished data) and also semisolid dosage form containing 0.5% and 1% dry extract were initially prepared (Cesca et al., 2012b). In search of an alternative to oral administration, in the present study, the dry extract was incorporated in the oral suspension due to its partial solubility in aqueous vehicle.

The *A. moluccanus* dry extract used in the present study was obtained on an industrial scale in the form of a powder with agglomerated and smooth particles, a greenish brown colour and characteristic odour. The average particle size was $129.388 \pm 62.907 \mu\text{m}$, moisture content was $4.04 \pm 0.05\%$ (Quintão et al., 2011) and the specification of total phenolic content expressed in swertisin is 3.04–3.72%.

Development of oral suspensions

In the suspensions development, besides the suspending agents used in the formulations shown in Table 1, xanthan gum, hypromellose and methylcellulose were also evaluated as viscosity agents, but were rejected due to the unfavourable organoleptic aspects obtained. Therefore, characterisation analyses were conducted for formulations I at X containing CS and MCCS. The suspensions showed flavour and colour characteristics of dry extract, i.e., greenish brown, not bitter in taste, agreeable odour, and homogeneous dispersion of extract in the vehicle.

The formulations showed a pH value of 4.46 at 5.17 (Table 2). The use of different suspending agents did not result in any significant changes in this property. The suspensions containing only water as vehicle (batches I, III, V and VII) had lower sediment volumes and also higher settling rates (Table 2 and Fig. 1). The sorbitol addition in the vehicle increased the sediment volume and the settling rate decreased, probably due to the increased viscosity provided by this vehicle.

The sediment of formulations was classified as flocculated or unflocculated from the data of the rate of settling after resting for 8 h, the sediment characteristics (loose or compact) and the aspect of the supernatant (cloudy or clear). The suspending agents used resulted in suspensions with smaller sediment volume, but which was kept loose. The formulation containing 0.5% CS had slower and more compact sediment than the formulation with 1% MCCS (Table 2). The suspensions showed redispersion times of less than

Table 2

Results of pH value and settling and rheological behaviours of suspensions containing *Aleurites moluccanus* dry extract.

Batches	pH	Rate of settling ^a (%)	Sediment characteristic	Supernatant characteristic ^c	Resuspension (min)	Viscosity (mPas s)	n^d
I	5.10 \pm 0.02	49.67 \pm 0.03	Compact	+	0.28 \pm 0.02	35.67 \pm 2.13	0.74 \pm 0.03
II	4.56 \pm 0.02	98.00 \pm 0.03	Loose	++	0.28 \pm 0.02	44.28 \pm 3.69	0.63 \pm 0.71
III	4.77 \pm 0.01	40.00 \pm 0.07	Loose	+++	0.19 \pm 0.01	25.83 \pm 3.69	0.19 \pm 0.02
IV	4.82 \pm 0.02	83.66 \pm 0.06	Loose	+++	0.33 \pm 0.02	38.13 \pm 2.13	0.98 \pm 0.10
V	5.06 \pm 0.01	39.66 \pm 0.00	Loose	++	0.06 \pm 0.01	38.13 \pm 2.13	0.18 \pm 0.02
VI	4.46 \pm 0.00	75.66 \pm 0.02	Loose	++	0.10 \pm 0.02	44.28 \pm 3.69	0.20 \pm 0.07
VII	4.81 \pm 0.00	25.66 \pm 0.00	Loose	++	0.08 \pm 0.01	38.13 \pm 2.13	0.25 \pm 0.02
VIII	4.83 \pm 0.01	39.00 \pm 0.06	Loose	+++	0.10 \pm 0.01	18.45 \pm 0.00	0.63 \pm 0.02
IX	5.17 \pm 0.03	nd ^b	Compact	+	0.61 \pm 0.02	53.20 \pm 1.99	0.91 \pm 0.08
X	5.08 \pm 0.03	33.00 \pm 0.04	Loose	++	0.05 \pm 0.00	6.45 \pm 0.18	0.24 \pm 0.02

^a Rate of settling after 8 h.

^b It did not observed the sediment formation.

^c + cloudy; ++ less cloudy; +++ clear.

^d Index of flow behaviour.

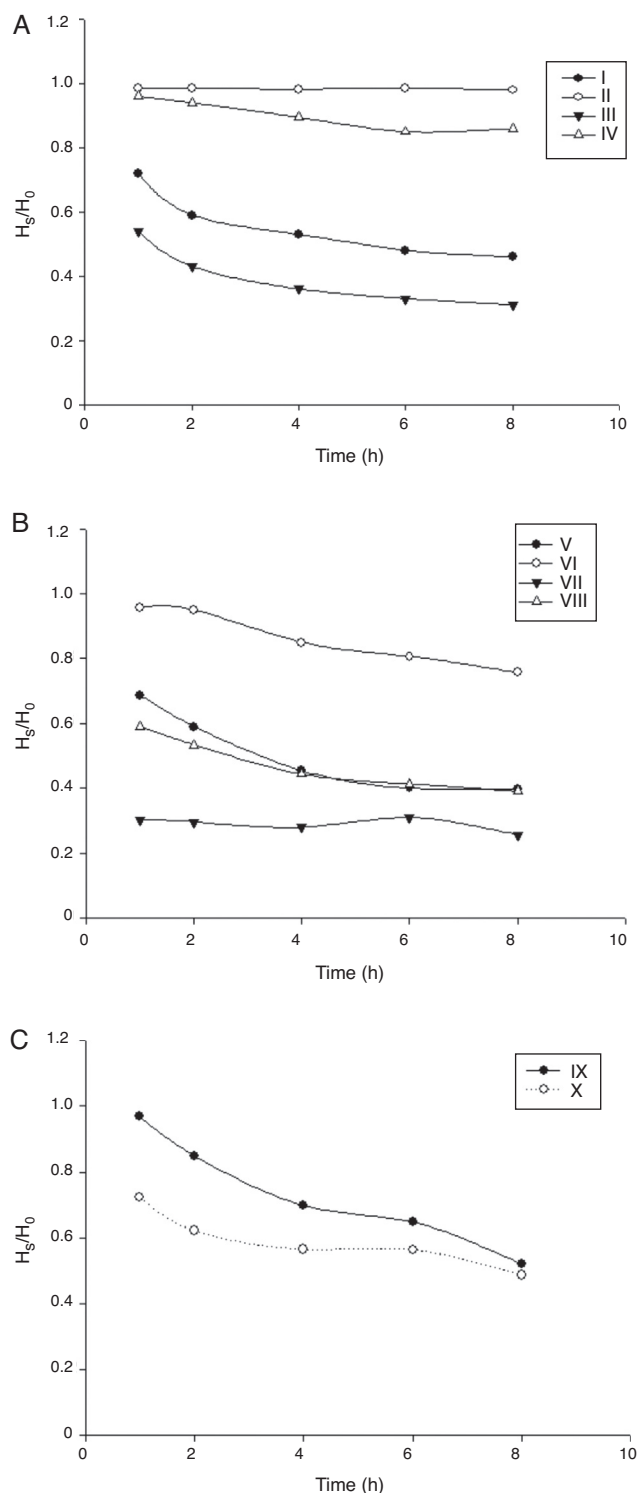


Fig. 1. Settling behaviour of suspensions containing dry extract of *Aleurites moluccanus* with different suspending agents. (A) Croscarmellose sodium; (B) mixture of carmellose sodium + cellulose; (C) sorbitol 20%.

30 s (Table 2). The behaviour was dependent on the suspending agent and vehicle. The formulations with MCCS and only water as vehicle showed the shortest redispersion time, which is related to the type of sediment formed.

The average viscosity of the suspensions was dependent upon the type and concentration of the suspending agent and vehicle. The addition of sorbitol as a carrier replacing part of the water (50%)

increased the viscosity of the formulations. These results have a direct relationship on the rate of settling and may have also influenced the redispersion time.

The suspensions exhibited non-Newtonian rheological behaviour, i.e., a reduction of viscosity with increasing shear rate (γ). Materials with non-Newtonian behaviour may exhibit plastic ($n=1$), pseudo-plastic ($n<1$) and dilatant ($n>1$) flow. To determine the flow type, in this study, we used the Ostwald-de Waele's mathematical model. As shown in Table 2, the suspensions have n value smaller than 1, therefore they have a pseudo-plastic flow-type.

The *A. moluccanus* suspensions showed different flow behaviour in the ascendant and down rheological curves. The thixotropic (positive thixotropy) or anti-thixotropic (negative thixotropy) behaviour was determined by quantifying the area between the upward and downward curves (Nutan and Reddy, 2010). This occurrence may be explained as a result of the increasing collision frequency of the dispersed particles or polymeric molecules in suspension, resulting in increased interparticle bonds over time (Martin, 2006). The rheological behaviour of the suspensions is expected for herbal suspensions in pharmaceutical use. They are prepared with sufficient viscosity to maintain the dispersed particles for a suitable time interval necessary for the administration of the medicine and reduced viscosity when shaken, allowing better homogenisation of the settled powder.

The total phenolic content expressed in the swertisin average assay and content uniformity of the IX and X formulations were determined by HPLC, being $103.3 \pm 0.69\%$ and $107.4 \pm 0.40\%$, respectively. The recommended range of total phenolic compounds is 90–110%; therefore, the suspensions are according to specifications. The RSD% was less than 2%, which is relevant because the dosage form is a heterogeneous system and is a phytoderivative.

Dose uniformity is a recommended test for pharmaceutical suspension assessment; thus, the content uniformity was performed to IX and X formulations. The total phenolic assay was determined with ten doses of suspension resulting in a content of $1.67 \text{ mg/ml} \pm 0.03$ (RSD%=2.18%) and $1.68 \text{ mg/ml} \pm 0.004$ (RSD%=0.28%) for IX and X suspensions, respectively. RSD% values less than 3% expressed the dose uniformity of suspensions and the safety of the drug.

For analysis of the dissolution profile and the dissolution test, suspensions of *A. moluccanus* were initially tested using different analyses, with the following variables being explored: dissolution medium (HCl buffer, pH 4.5 acetate buffer and pH 6.8 phosphate buffer), and stirring speed (50, 75, and 100 rpm).

The dissolution profiles obtained under different conditions of dissolution analysis showed that more than 80% of the total phenolic dissolved in the first three min of analysis (Fig. 2). We observed a higher dissolution rate in a simulated gastric environment (0.1 M HCl) and pH 4.5 acetate buffer in comparison to pH 6.8 phosphate buffer; thus, the acetate buffer was chosen as the dissolution medium. These results are in agreement with the dissolution medium selected for *A. moluccanus* tablets analysis (unpublished data). A weak influence of the stirring rate was observed on the proportion of dissolved phenolic compounds (Fig. 2) and there was no direct correlation between them. The speed of 75 rpm was chosen for further study.

Stability of suspensions

The stability study of suspensions was carried out with batches IX and X. The suspensions showed colour, odour and flavour characteristics that were unchanged after 180 days of storage at 40 °C. At time zero, and throughout the stability study, the suspensions showed a pH value of about 5.0 (Table 3). Although the change

Table 3Results of accelerated and long time stability study of *Aleurites moluccanus* suspension.

Analysis	Batches			
	IX		X	
	t_0	t_{180}	t_0	t_{180}
30 °C (long time study)				
pH	5.17 ± 0.03	5.03 ± 0.01	5.08 ± 0.03	4.93 ± 0.11
Redispersion time (s)	36.77 ± 1.26	11.33 ± 1.52	3.16 ± 0.23	2.60 ± 0.57
Viscosity (mPa s)	53.20 ± 1.99	20.5 ± 1.42	6.45 ± 0.18	3.69 ± 0.0
Zeta potential	-33.90 ± -33.90	-41 ± 4.12	-33.5 ± 5.79	-35.1 ± 5.77
TP ^a (mg/ml)	1.70 ± 0.04	1.40 ± 0.004	1.68 ± 0.007	1.44 ± 0.011
TP ^a dissolved (%)	1.58 ± 0.04	1.37 ± 0.05	1.54 ± 0.03	1.38 ± 0.004
40 °C (accelerated study)				
pH	5.17 ± 0.03	4.93 ± 0.02	5.08 ± 0.03	4.92 ± 0.03
Redispersion time (s)	36.77 ± 1.26	8.33 ± 2.56	3.16 ± 0.23	2.00 ± 0.50
Viscosity (mPa s)	53.20 ± 1.99	8.61 ± 1.73	6.45 ± 0.18	9.84 ± 2.13
Zeta potential	-33.90 ± -33.90	-37.7 ± 4.65	-33.5 ± 5.79	-33.3 ± 5.41
TP ^a (mg/ml)	1.70 ± 0.04	1.46 ± 0.02	1.68 ± 0.007	1.47 ± 0.03
TP ^a dissolved (%) ^b	1.58 ± 0.04	1.37 ± 0.03	1.54 ± 0.03	1.32 ± 0.02

^a TP: total phenolic expressed in swertisin.^b Total phenolic dissolved in 10 min.

was small, the values were considered statistically different with $p < 0.05$ in the analysis using F test at a significance level of 5%.

The formulation containing CS (IX) did not showed settling within 8 h, at the beginning and after 180 days. The formulations showed an increase in the rate of settling during the study, i.e., lower sediment volume. This behaviour is related to the more compact cake formation over the storage period. However the sediment formed was easy to redisperse. As shown in Table 3, formulations containing MCCS had faster rates of settling but also redispersed more easily. According to ANOVA analysis, formulation IX showed a significant difference in redispersion throughout the stability study. This difference was confirmed using the Tukey test between the times 30, 90 and 180 days compared to zero time. During the stability study, average viscosity was decreased (Table 3). These results are related to the behaviour of settling and redispersion, due to the lower viscosity, settling rate and faster redispersion.

The suspensions showed negative zeta potential (Table 3), with values below -30 mV, that is resultant of anionic suspending agent used, and this condition remained until the end of the stability study. Zeta potential values ± 30 mV or more are considered appropriate for keeping the particles dispersed in the dispersion vehicle.

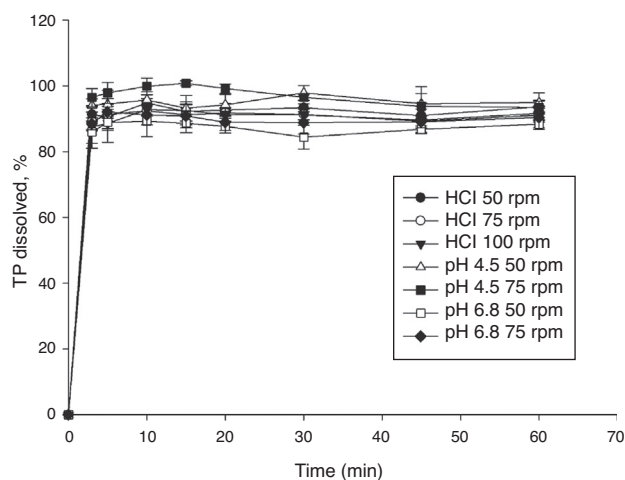


Fig. 2. Dissolution profile of total phenolic (TP) from *Aleurites moluccanus* suspensions in different analyses conditions (medium and speed), using paddle apparatus at 37 °C.

The formulations showed a significant decrease in total phenolic assay at end of the stability study. The decrease was 14.11% and 12.5% of total phenolic for formulations IX and X, respectively. In this study, the total phenolic content expressed in swertisin was employed as a parameter for monitoring the chemical stability of the extract. Taking into account the complexity that is inherent in chemical extracts and consequently herbal medicine, this variation is considered acceptable. Following Brazilian rules, the allowed active marker variation during stability study for a phyto-medicine should be within $\pm 15\%$ (Anvisa, 2012). It is necessary to evaluate other parameters/methods or applications of biological activity assays in order to monitor more broadly the stability of the extract (EMA, 2006). Furthermore, there are no chemical stability adjuvants, such as antioxidants, in the studied formulations. Being an extract with high content of phenolic compounds, chemical degradation by oxidation can occur and the marker content stability might increase with the addition of preservatives in the formulation, such as antioxidants and chelating agents.

The stability study was also accompanied by the dissolution test of the suspensions at zero and 180 days. The proportion of TP dissolved in 10 min was determined. As shown in Table 3, the formulations at time zero showed that more than 90% of TP dissolved in 10 min. This result is similar to that obtained for the formulations in the development stage.

Validation of the HPLC method

The analytical methods for total phenolic expressed in swertisin for the assay and dissolution method were successfully validated according to recommendations for the validation of analytical methods (ICH, 2005; Anvisa, 2012).

The specificity of the method is shown in Fig. 3. In the chromatographic profile of vehicle, only one peak was observed in 32 min; this same peak was found in the chromatographic profile of the suspension containing extract, but without interference in retention time of peaks selected for quantification of the phenolic compounds present in the extracts (highlighted in Fig. 3). The resolution (R) between the 2''-O-ramnosilswertisin neighbour peak was 1.595, and between swertisin and 2''-O-ramnosilswertisin was 1.384, showing that the principal peaks have $R > 1.0$; i.e., the method provides adequate separation of these peaks.

The linearity of the method was determined for the pure extract and the extract added to the suspension. The method was linear from 0.5 to 5.0 mg/ml (in dried extract) on both curves, with a cor-

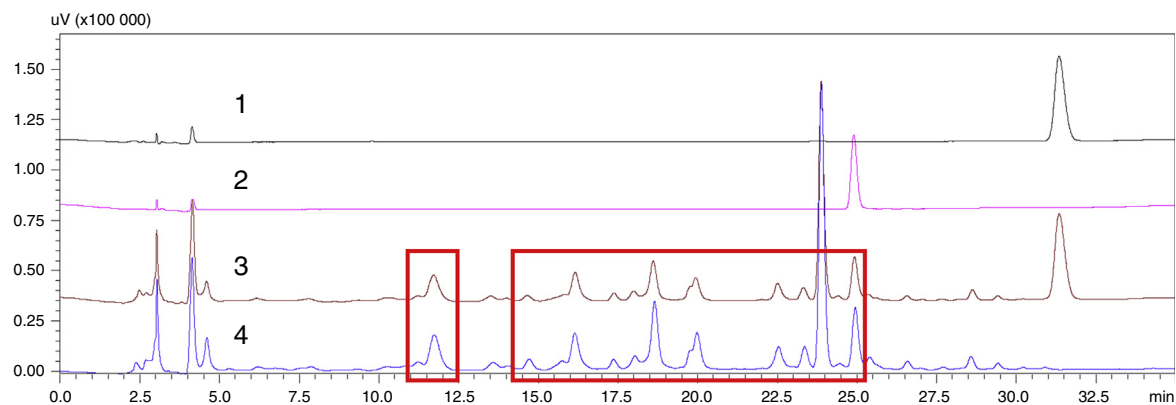


Fig. 3. Chromatography profile of suspending vehicle (1); swertisin (2); suspension containing dry extract of *Aleurites moluccanus* (3) and dry extract of *A. moluccanus* (4) by HPLC.

Table 4
Results of accuracy and precision of analytical method to assay of suspension containing *Aleurites moluccanus* dry extract.

Recovery				
Theoretical TP added (μg/ml)	Practical TP added (μg/ml)	Recovery (%)	Average (%)	% RSD
19.26	22.34	116.00	103.13	10.82
32.10	30.83	90.06		
44.94	43.74	97.34		
Repeatability and Intermediary precision ^a				
	Analyst 1	Analyst 2	Intermediary precision	
TP expressed in swertisin (mg/ml)	1.54	1.62	1.58	
RSD%	2.24	1.37	3.37	

^a Analysis in 6 replicates.

relation coefficient greater than 0.99. Also, the curves were parallel (slope of 2.10⁰⁶), showing that there was no matrix interference in the linearity of the quantification method of TP in the range of concentrations analysed.

The analytical method showed accurate intra-assay results, with an RSD% of 1.71% (Table 4). In the intermediate precision with another analyst on at least 2 different days, the RSD% was 2.18%. Assessing the intermediate precision, for two days of analysis, the

Table 5
Results of analytical validation parameters of dissolution method of suspensions containing *Aleurites moluccanus* dry extract.

Linearity					
Intercept	R	F _{calc}	F _{crit}		
−41,648.99	0.9967	1510.53	2.12 × 10 ^{−07}		
Recovery					
Batches	IX		X		
Theoretical TP added (μg/ml)	Practical TP added (μg/ml)		Recovery (%)		
4.94	5.31	107.60	5.25	106.38	
8.92	8.92	100.01	9.15	102.58	
12.82	13.22	102.74	12.35	95.99	
Average %	103.45		Average %	101.65	
RSD%	3.71		RSD%	5.16	
Repeatability and intermediary precision ^a					
	IX		X		
	Analyst		Analyst		Intermediary precision
	1	2	1	2	
TP assay (mg/ml)	1.69	1.52	1.60	1.65	1.53
DPR%	2.96	2.54	7.48	1.78	1.57

^a Analysis in 6 replicates.

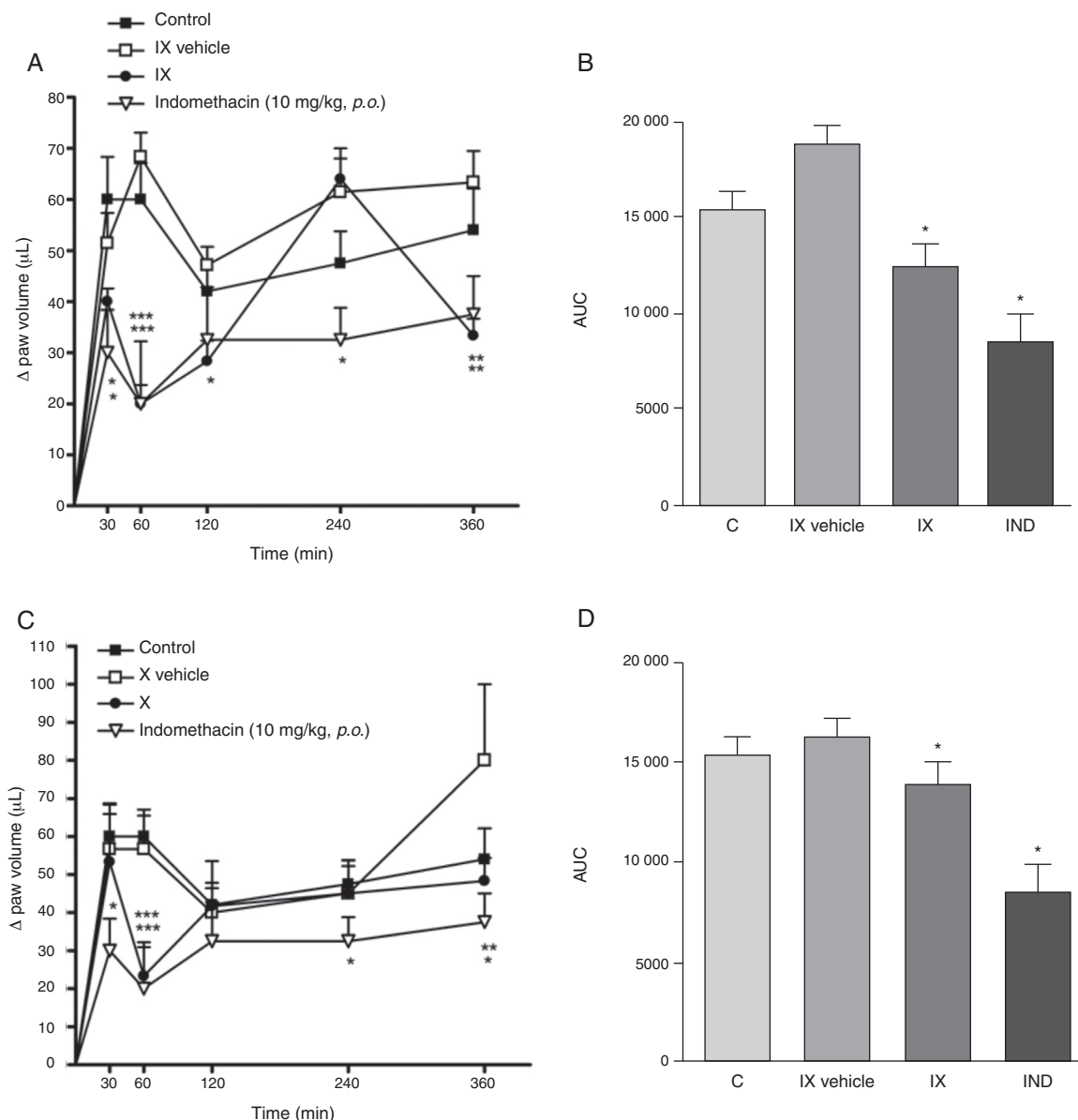


Fig. 4. Results of anti-inflammatory activity of suspensions containing *Aleurites moluccanus* dry extract using paw oedema induced by carrageenan (300 μg/paw) in mice. Saline solution as negative control and indomethacin (10 mg/kg p.o.) as positive control.

RSD% was 3.05%, which was lower than that recommended by ANVISA, which is up to 15% for bioanalytical samples (Anvisa, 2012).

The average recovery of TP in the formulation IX was 103.1% (Table 4); therefore, this is within the range of 90 to 108% recommended for concentrations between 1 and 10% (AOAC, 2012), when considering that suspensions have 5% of dried extract.

The method was robust, with relatively low RSD% to changes in temperature and flow of the mobile phase. Although changes occurred in the retention time and resolution between the two main markers with flow changes, the TP content showed no significant changes with small, deliberate changes in the method (data not shown).

The method was developed and selected for analysis of the dissolution profile and the dissolution test of the suspensions of *A. moluccanus* was validated following the recommendations of RE 899 (Anvisa, 2003). In this analysis, formulations IX and X were used.

The specificity of the dissolution method was assessed by comparing the chromatograms of suspending vehicles, suspensions containing dry extract and pure extract. The

chromatogram profiles were similar to the assay method (Fig. 3).

The results for the validation of the dissolution method are shown in Table 5. The method was linear from 0.24 to 0.82 mg/ml, with a correlation coefficient of 0.9967. The dissolution test was precise for intra-assay variation, with an RSD% of 2.96 and 2.54% for formulations IX and X, respectively. These were lower than the maximum recommended by Anvisa, which is 5% and 15% for analytical and bioanalytical methods, respectively (Anvisa, 2003; 2012). In intermediate precision with another analyst on at least 2 different days, the RSD was 1.78% and 1.57%, respectively, which is also in accordance with the recommendations of ANVISA. Comparing the two tests, precision was obtained with an RSD% of 7.48% and 4.87% for the formulations IX and X, respectively. The RSD of 7.48% is within that recommended by ANVISA of up to 15% (Anvisa, 2012), and is relatively high due to the heterogeneity in this dosage form. The average recovery of formulations IX and X was 103.45% and 101.65% respectively. These values are within the range recommended by the AOAC of 80–110% (AOAC, 2012). The LOQ for the method was 0.143 mg/ml.

Analysis of the pharmacological activity in vivo

The pharmacological experiments carried out with the formulations IX and X validated the anti-inflammatory activity of the dry standardised extract of *A. moluccanus* when administered orally as a suspension. All formulations were able to significantly reduce the paw oedema induced by carrageenan. Fig. 4 demonstrates that the formulation IX (containing CS) showed a reduction in paw oedema, with $35.5 \pm 7.6\%$ of inhibition. Otherwise, formulation X (containing MCCS) did not interfere with the effects of carrageenan in the mice paws. The positive-control drug used, indomethacin, was able to reduce paw-oedema, with an inhibition of $54.8 \pm 11\%$.

Conclusion

The pharmaceutical suspensions containing 50 mg/ml of dry extract of *A. moluccanus* were obtained and physical and physico-chemistry properties of formulations were appropriate and characteristic of the dosage forms. The use of carmellose sodium as a suspending agent and sorbitol as a vehicle was important for the organoleptic and physical properties. The suspension-containing extract showed pharmacological activity observed in previous studies using extract only.

The analytical methods used in the assay and dissolution test were linear, accuracy, precise and selective for phenolic compounds present in the formulations.

The liquid dosage form developed can be used as an alternative to tablets for the oral administration of new anti-inflammatory and analgesic herbal medicines.

Authors contributions

RMD and SSC carried out the laboratory work, data analysis and writing of the manuscript. NLMQ supervised the pharmacological studies and contributed to critical writing and reading of the manuscript. CMS and VCF contributed to botanical, phytochemistry and analytical studies. TMBB and RMLS contributed to designed the study, supervised the analytical and technological studies and contributed to critical writing and reading 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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