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STABILITY INDICATING HPLC METHOD FOR DETERMINATION OF MYRSINOIC ACIDS A AND B IN *RAPANEA FERRUGINEA* EXTRACTS AND NANOEMULSIONS

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Keywords:

HPLC. Stability indicating method. Myrsinoic acid A. Myrsinoic acid B. Nanoemulsions. *Rapanea ferruginea*

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ABSTRACT: *Rapanea ferruginea* Mez. stem barks and fruits extracts have demonstrated anticholinesterasic and antimicrobial activities assigned to myrsinoic acids A and B (MAA and MAB), compounds that have also proven anti-inflammatory and antinociceptive activities. To stablish the quality control of nanotechnology-based herbal medicines, the aim of this study was develop and validate a stability indicating HPLC–DAD method for quantitative analysis of MAA and MAB markers in *R. ferruginea* stem barks and fruits extracts and nanoemulsions. Chromatographic separation was conducted on a Kinetex®C_{18} column (150 x 4.6 mm x 2.6 µm), with mobile phase consisted of acetonitrile: methanol: water (acidified with H_3PO_4 pH 2.50) and eluted in gradient mode with flow rate of 0.9 mL min^{-1}, with 30 min of analysis and detection at 260 nm and 270 nm. The method was specific, linear, sensitive, accurate and robust for both markers. The chromatograms of stem barks and fruits extracts submitted to forced degradation study (acid, oxidative, UV and visible light) did not show impurities interference in MAA and MAB quantification, therefore, the method was considered stability indicative. The developed method has been successfully used to quality control of *R. ferruginea* extracts and nanoemulsions containing these extracts and applied to the stability study of nanoemulsions.

INTRODUCTION: *Rapanea ferruginea* Mez., popularly known as "capororoca", occurs in Bolivia, Mexico, Argentina, Paraguay, Uruguay and Brazil, being particularly frequent in the atlantic forest [1]. Phytochemical analysis performed with *R. ferruginea* allowed the identification and isolation of myrsinoic acids A (MAA), B (MAB) and C (MAC), which has been awarded important anti-inflammatory activity [2, 3].

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Previous studies have demonstrated a potent anticholinesterasic(unpublished data) and selective antimicrobial activity against gram positive bacteria [4] of MAA and MAB compounds, as well as *R. ferruginea* stem barks and fruits hydroalcoholic extracts. The *R. ferruginea* fruits extract [4] and its major constituent MAA were active on memory (acquisition, consolidation and evocation) of normal mice and animals with induced Alzheimer (unpublished data). Due the important activity against *L. brasiliensis*, Cechinel-Filho et al. [5] first suggested the ethanolic extracts of stem barks of *R. ferruginea* and MAB as possible source of antileishmanicidal agents. MAB also showed antinociceptive activity [6, 7].

Herbal formulations reached high acceptability as therapeutic agents for various diseases [8, 9]. Currently, nanotechnology used for medicinal and isolated active substances plants has shown promising results to obtain desired therapeutic effects [10, 11]. The increased solubility, stability, bioavailability and pharmacological activity of various extracts from popular herbs using nanosystems as polymeric nanoparticles, nanospheres, nanocapsules, liposomes, solid lipid nanoparticles and nanoemulsions have been reported in the literature. The advantage of phyto-derived nanosystems includes improvement in the solubility of the active components, bioavailability and pharmacological action, chemical and physical stability, in addition to stability in biological environment, or minimizing the extensive first-pass metabolism, protection against the toxicity, improving the distribution of macrophages tissue, sustained release, physics and chemical protection against degradation, and also contributing to increased patient compliance to its use [10-12].

Seeking improvements in its pharmacological activities, studies conducted by our research group aimed the development of nanoemulsions containing *R. ferruginea* extracts (bark and fruits) targeted to the treatment of infectious and inflammatory skin processes (unpublished data).

For quality guarantee of herbal medicines at all stages of manufacture and storage, becomes necessary to establish appropriate analytical methods and technologies [13]. The ICH guideline Q1A on Stability Testing of New Drug Substances and Products [14] emphasizes that features changes in pharmaceutical products can influence its quality, safety and efficacy, must be achieved through validated stability indicating testing methods.

According to FDA [15], degraded samples obtained from stress studies must be applied to develop stability indicating and specific methods, without interference of impurities and excipients in quantification of the active ingredient [16-18]. Natural products are not included in this requirement, due to the complexity of the plant matrix. However, it is necessary to know the profile of such materials stability as well as the application of stability indicating methods for the intermediates and compositions based on plant extracts. Therefore, aiming the quality control of *R. ferruginea* herbal extracts and nanotechnology herbal medicines, the objective of the present study has been develop and validate a gradient stability indicating HPLC–DAD method for quantitative analysis of MAA and MAB markers in *R. ferruginea* stem barks and fruits extracts and nanoemulsions.

MATERIALS AND METHODS:

Reagents and standards

For HPLC analysis all solvents used were HPLC grade (Tedia, Fairfield, Ohio, USA). The water was purified using a Milli-Q system (Millipore, Massachusetts, USA). All solutions were filtered through 0.45 µm membrane (Millipore, Massachusetts, USA). Sodium hydroxide and hydrochloric acid were from Dinâmica® (São Paulo, Brazil) and 30% hydrogen peroxide was purchased from Vetec® (Rio de Janeiro, Brazil). Myrsinoic acids A and B were isolated from a mixture of hydroethanolic extracts of stem barks, branches and leaves of *R. ferruginea* (43g), performed according to Bella-Cruz et al.[4] and Hess et al. [6]. The compounds were structural characterized by Infra-Red Spectroscopy (FTIR), HPLC and Nuclear Magnetic Resonance (^{1}H NMR and ^{13}C NMR). MAA and MAB showed purity of 76.2% and 98.9%, respectively, by HPLC.

Plant Material:

Stem barks and fruits of native specimens obtained from vegetative propagation of authentic *R. ferruginea* were collected in Blumenau (Santa Catarina, Brazil) in April/2013 and identified by Prof. Oscar Benigno Iza (UNIVALI, Itajaí-SC, Brazil). Stem barks were collected from a 35 cm diameter tree, from 20 cm above soil. Fruits selected were in mature and immature stages. Plant materials were dried in oven with air circulation at 35 °C, for seven days, powdered and sieved. A voucher specimen was deposited at the Barbosa Rodrigues Herbarium (Itajaí-SC, Brazil) under number HBR 52715.

Preparation of hydroalcoholic extracts:

The stem barks hydroethanolic extract (extractive solution and extract) was prepared by dynamic maceration with ethanol: water 90:10 (v/v) at a plant: solvent ratio of 10% (w/v), stirred for 6 h at

330 rpm. Thereafter the extract was filtered through three layers of Sontara®. The extraction solution was filtered again under vacuum filter paper obtaining the extractive solution, according standardized by Baccarin et al. [19]. The extractive solution was then reduced to 50% of its volume in a water bath at 45 °C followed by concentration in a circulating air oven at 45 ° C to provide a syrupy appearance obtaining the extract. The dry residue was 0.48 ± 0.02 % and 51.17 ± 0.53 % for the extractive solution and the extract, respectively.

Fruits hydroethanolic extracts were prepared by dynamic maceration with ethanol:water 90:10 (v/v), at a plant: solvent ratio of 5% (w/v), stirred for 5 h at 300 rpm and then filtered as described to stem barks extracts. To obtain the extract, the solution was kept in water bath at 45°C. The dry residue was 0.72 ± 0.14% for extractive solution.

Preparation of nanoemulsions:

Oil/water nanoemulsions of *R. ferruginea* stem barks and fruits extracts were prepared by low energy emulsification method, briefly described below. Optimized nanoemulsion containing 0.13% of stem barks extract (NSB) was prepared with isopropylmyristate as oil phase, Alkest® CSO 400 and Span 80® as surfactants and distilled water as an aqueous phase. Nanoemulsion formulation containing 0.46% of fruits extract (NF) was prepared with Polymol® as oil phase, Span 80® and Ultramona® RH400 as surfactants and distilled water as an aqueous phase. The dry residues were considered for estimating the extracts content incorporated in nanoemulsions

Equipment and chromatographic conditions:

Chromatographic analysis were carried out on a HPLC Shimadzu® LC 20-AT system, consisting of a quaternary pump and a Shimadzu SPD-M20A photo diode array detector, SIL-20A HT auto-sampler, with a Shimadzu CTO-10AS VT column oven equilibrated at 35 °C, and software LC Solution was used. The chromatographic column used was Kinetex® C_{18} column (150 x 4.6 mm) with core-shell particles of 2.6 µm. The separation was achieved on a gradient method. The mobile phase consisted of methanol:acetonitrile:water (pH 2.5, phosphoric acid) at a flow rate of 0.9 mL min^{-1}. The gradient elution was programmed as follows: 0-2 min (25:5:70), 2-5 min (25:30:45), 5-10 min (25:60:15), 10-12 min (20:70:10), 12-15 min (15:75:10) and 15-20 min (15:84:1) than return to the initial condition until 30 min of analysis. The analysis was monitored at 260 nm for MAA and at 270 nm for MAB.As described by Zermiani et al. [20] for isolated MAA and MAB.

Preparation of standard and sample solutions:

Working solutions were prepared weighing 1 mg of each compound (MAA and MAB), taking into account its potency previously determined by HPLC, which were transferred to 10 mL volumetric flasks and dissolved with methanol (100 µg mL^{-1}) using ultrasonic vibrator for 10 min.

Sample solutions of stem barks and fruits extractive solutions were prepared by dilution at 1:5 and 1:10 in methanol, respectively.

Sample solutions of the nanoemulsions containing extracts of the barks and fruit *of R. ferruginea* were prepared by dissolution of 0.5 g of nanoemulsions in a volumetric flask of 5.0 mL, to which was added about 2.5 mL of acetonitrile and agitated in ultrasound for 20 min. After made to up the flask volume with the same solvent, the sample was centrifuged at 3000 rpm for 10 minutes.

All the solutions were filtered through a 0.45 µm cellulose regenerated membrane filter before injection into HPLC.

HPLC Method validation:

The method was validated according to the ICH guidelines [21]. The selectivity of the method was evaluated through the resolution (*R*) between MAA, MAB and other peaks in stem barks and fruits extracts chromatograms. The selectivity was also determined through comparison of nanoemulsions blank with nanoemulsions containing the stem barks and fruits extracts. The peak purity was accessed for MAA and MAB in each sample using the PDA detector. A forced degradation study was performed to verify the stability indicating power of the analytical procedures used, as described below

Linearity was determined injecting three times nine solutions at concentrations of 1, 5, 10, 25, 50, 75,

100, 120 and 150µg mL^{-1} of MAA and MAB. The simple linear regression was calculated from MAA and MAB peak areas (mAU) versus concentration by the least-squares method. Limits of detection (LOD) and quantification (LOQ) were determined by the signal-noise relationship of 3:1 and 10:1, respectively. The evaluation of accuracy was performed through the analyte recovery test in triplicates, adding MAA and MAB at 25, 50 and 75 µg mL^{-1} to stem barks extractive solution (prepared as described above).

Repeatability (intra-day precision) was determined through analysis of six solutions of stem barks and fruits extractive solutions and through analysis of six samples solutions of each nanoemulsion. The relative standard deviation (RSD) of MAA and MAB assay were determined.

Robustness of the method was evaluated by changing the mobile phase flow (0.8, 0.9 and 1.0 mL min^{-1}), oven temperature (34, 35 and 36 ºC) and pH of acidified water (2.5, 2.6 and 2.7). Variation in retention times, areas and MAA and MAB assays in the samples (stem barks and fruits hydroethanolic extracts) were evaluated using analysis of variance (ANOVA) single factor ($p < 0.01$).

Stress degradation studies:

In order to develop a gradient stability indicating LC–UV method a forced degradation study was conducted. Stem barks and fruits extracts were submitted to stress tests under conditions of acid (1 mol L^{-1}HCl, 24 h, 25 °C) and oxidative hydrolysis (30% H_2O_2, 6 h, 25 °C). Sample aliquots of 10 mg were dissolved with 2.5 mL of methanol, sonicated for 10 min and diluted with degrading agent (1:1 v/v) to 1 mg mL^{-1}. After the degradation processes, 500 µL of each solution was transferred to a 1 mL volumetric flask, neutralized when necessary and the volume was completed with methanol. The extracts were also submitted to visible light (2.4 mi lux h^{-1}) and UVA (400 Whm^2) irradiation in a photostability chamber (Mecalor®, São Paulo, Brazil). After exposure, 10 mg of each extract was diluted in 10 mL of methanol, submitted to sonication for 30 min. All the solutions were filtered through a 0.45 µm cellulose regenerated membrane filter and analyzed employing the HPLC method. Assay of stressed samples were performed by comparison with not degraded reference sample prepared at the same theoretical concentration.

HPLC Method application:

For MAA and MAB assay determination, *R. ferruginea* stem barks and fruits extracts were diluted at 1 mg mL^{-1} and submitted to sonication for 30 min. The solutions were filtered through a 0.45 mm cellulose regenerated membrane prior to injection into HPLC.

The MAA and MAB assay of the nanoemulsions containing stem barks and fruits extracts (NSB and SF, respectively) were determined by dissolving 0.5 g of each formulation in 2.5 mL of acetonitrile, in a 5 mL volumetric flask, submitted to sonication for 20 min. Then, the volume was completed and the resulting solution was centrifuged at 3000 rpm for 10 min. The supernatant was filtered through a 0.45 µm cellulose regenerated membrane and analyzed by using the above-described HPLC method.

Accelerated stability studies of nanoemulsions were performed by keeping the samples at room temperature (25 ± 2 °C) and at 40 ± 2 °C, in amber flask. The nanoemulsions were evaluated freshly prepared and after 1, 3 and 6 months and after 1 and 2 months for stem barks extract (NSB) and for fruits extracts (NF), respectively. Quantification of MAA and MAB in the formulation samples was conducted using the established HPLC method. Freshly prepared samples were used as reference.

RESULTS AND DISCUSSION:

Method validation:

When applied for the analysis of *R. ferruginea* stem barks extract, the HPLC-UV method showed satisfactory peaks resolutions *(R)* of 2.98 and 1.39, for MAA (1) and MAB (2), respectively (**Fig. 1a**). In the fruits extract chromatogram (Fig. 1b) was observed only the MAA (2) (R = 10.16) and another peak (3) in the less polar region of chromatogram, which according previous research may be a triglyceride, whose chemical structure has not been fully elucidated, other major constituent of *R. ferruginea* fruits (unpublished data). Peak purity through PDA was > 99.99% for MAA and MAB.

The developed method was also selective to nanoemulsions analysis. There was no interfering peak in the retention times of MAA and MAB as can be seen in the NSB and NF blanks chromatograms of nanoemulsions (**Fig.1c** and **1d).**

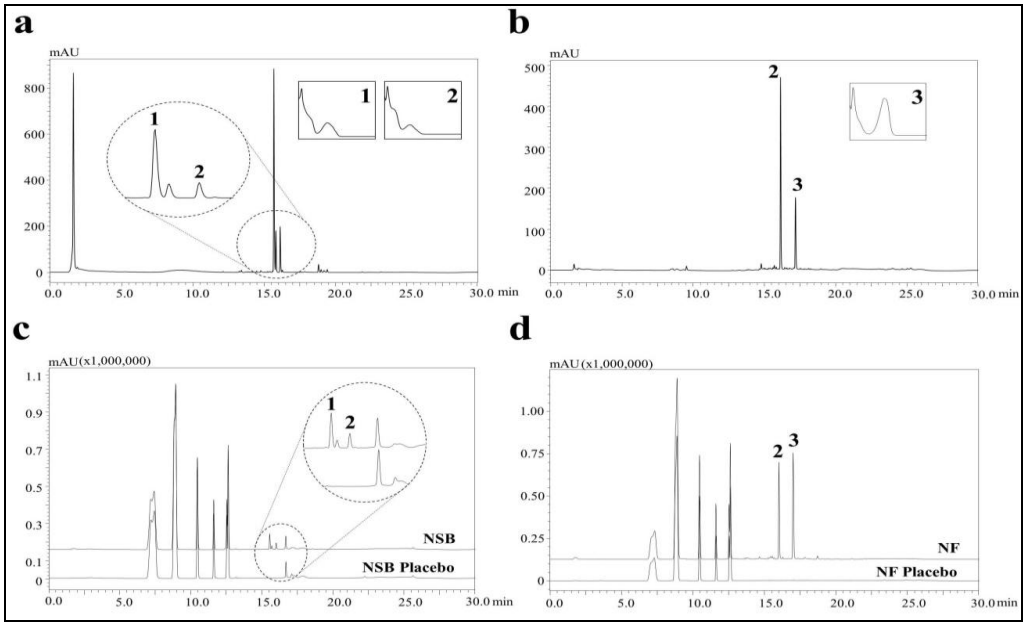

FIG. 1: CHROMATOGRAPHIC PROFILES OF: (a) STEM BARKS EXTRACTIVE SOLUTION DILUTED AT 1:5 AT 270 nm (MAA AND MAB UV SPECTRA IN THE INSERT); (b) FRUITS EXTRACTIVE SOLUTION DILUTED AT 1:10 AT 260 nm (PEAK 3 UV SPECTRA IN THE INSERT); CHROMATOGRAPHIC PROFILE COMPARISON OF: (c) NANOEMULSION CONTAINING 0.25% OF STEM BARKS EXTRACT (NSB) AND BLANK (NSB BLANK); (d) NANOEMULSION CONTAINING 1% OF FRUITS EXTRACT (NF) AND BLANK (NF BLANK).

The MAA and MAB standard calibration curves exhibited an excellent linearity and good correlation coefficients over the given range of 1 – 100 µg mL^{-1}. The mean linear regression equation from three calibration curves for MAA was $y = 28082x - 1880$ ($r^2 = 0.9994$). The F test statistic (F) indicates a good linearity for the model, once F_{calc}(7705.79) was higher than F_{crit} (3.64×10^{-9}). The MAB mean linear regression equation obtained for the cited concentration range was $y = 51940x + 6217.2$ ($r^2 = 0.9996$). Statistical analysis of linear regression demonstrates the statistical significance of the linearity of the method (F_{calc}11204.99 and $F_{crit}1.43 \times 10^{-9}$).

The sensitivity of the method for MAA and MAB was expressed by the signal-noise relationship. The LOD and LOQ were found to be 0.05 and 0.5 µg mL^{-1} for MAA, respectively. For MAB the values were 0.01 and 0.05 µg mL^{-1} for LOD and LOQ, respectively. Accuracy was assessed by the standard addition method at three levels: 25, 50 and 75 µg mL^{-1}. The overall recovery percentage was found to be 98.6 % for MAA and 96.9 % for MAB. The % CV observed in all determinations were < 3 % for both compounds (**Table 1).** Hence, the method showed good accuracy at low, medium and high level concentrations for the markers.

TABLE 1: ACCURACY RESULTS, EXPRESSED IN PERCENTAGE OF RECOVERY, FOR DIFFERENT LEVELS OF MAA AND MAB SPIKED IN STEM BARKS EXTRACTIVE SOLUTIONS.

Constituent	Spiked (µg mL^{-1})	Found (µg mL^{-1})* (%CV)	Recovery (%)	Mean (%) (%CV)
MAA	26.45	26.37 (0.28)	99.70	98.56 (2.44)
	52.90	50.68 (0.09)	95.80	
	78.03	78.17 (0.29)	100.18	
MAB	26.66	26.35 (0.30)	98.84	96.89 (2.29)
	53.33	50.38 (0.06)	94.47	
	79.99	77.87 (0.31)	97.35	

*triplicate injection.

Table 2 presents the results of precision (repeatability) of the method. The repeatability was found to be <3% for MAA and MAB in all samples. Such results indicate that the method proposed presents good precision when applied to stem barks and fruits extractive solutions and nanoemulsions analysis.

TABLE 2: SUMMARY OF PRECISION (REPEATABILITY) RESULTS FOR MAA AND MAB IN STEM BARK AND FRUITS EXTRACTIVE SOLUTIONS AND NANOEMULSIONS ANALYSIS.

Sample	Mean measured concentration (µg mL^{-1})* (%CV)	
	MAA	MAB
Stem barks extractive solution	30.98 (0.43)	54.38 (0.41)
Fruits extractive solution	54.46 (2.88)	-
SBN	6.04 (1.86)	5.54 (1.39)
FN	61.63 (1.93)	-

*n=6; SBN = stem barks extract nanoemulsion; FN = fruits extract nanoemulsions.

Statistically significant differences (F_{calc} higher than F_{crit}) were observed in retention times (Rt), areas and MAA and MAB assays in the samples for deliberate variations in the mobile phase flow (0.8, 0.9 and 1.0 mL min^{-1}), oven temperature (34, 35 and 36 ºC) and pH of acidified water (2.5, 2.6 and 2.7). Although, the method can be considered robust due to the low % CV measured for MAA in all tested parameters. The present method showed to be robust for the assay of MAB present in almost conditions. The exception was the increase in flow rate, that resulted in a decrease of Rt, Area, and assay of MAB, with % CV > 3%. Therefore, the mobile phase flow must be set precisely, for consistent results to MAB quantification (**Tables 4 – 6** at supplementary material).

Stress Degradation Studies:

The results of MAA and MAB residual contents (% in relation to the original assay, in the non-degraded sample) in extracts after acid, oxidation, UVA and visible light degradation are shown in **Table 3**.

TABLE 3: IN STEM BARK EXTRACT WAS OBSERVED A MAJOR LABILITY OF MAA THAN OF MAB IN ALMOST STRESS CONDITIONS TESTED, EXCEPT AT THE UVA EXPOSURE. THE MAA PRESENT IN FRUITS EXTRACT SHOWED HIGHER DEGRADATION WHEN COMPARED WITH THE SAME COMPOUND IN STEM BARK EXTRACT

Parameters	Degradation (%)		
	Stem barks extract		Fruits extract
	MAA	MAB	MAA
Acid (1 mol L^{-1} HCl, 24 h)	23.68	8.89	32.34
Oxidative (30% H_2O_2, 6 h)	4.34	0	42.26
UVA (400 Whm^2)	7.43	11.42	46.41
Visible light (2.4 mi lux h^{-1})	0.95	0	98.66

TABLE 4: CHROMATOGRAPHIC PARAMETERS IN THE ROBUSTNESS TESTS OF THE HPLC METHOD FOR MYSINOIC ACID A (MAA) ASSAY IN *R. FERRUGINEA* STEM BARKS EXTRACTIVE SOLUTION.

Parameter	Average (% CV_{intra})		
	Rt	Area	Assay (mg/g)
Mobile phase flow			
0.8 mL min^{-1}	17.10 (0.03)*	583742 (0.53)	12.65 (0.54)
0.9 mL min^{-1}	16.,23 (0.04)*	578656 (0.23)	12.54 (0.23)
1.0 mL min^{-1}	15.38 (0.06)*	566824 (0.32)*	12.28 (0.33)
DPR (%)	5.29	1.5	1.53
F_{calc}/F_{crit}	9379.80	8.93	8.93
Oventemperature			
34 ºC	16.11 (0.09)*	571825 (0.18)	12.39 (0.18)
35 ºC	16.23 (0.04)*	578656 (0.23)*	12.54 (0.23)*
36 ºC	16.06 (0.04)*	572996 (0.17)	12.42 (0.18)
DPR (%)	0.52	0.64	0.65
F_{calc}/F_{crit}	41.19	6.07	6.07
pH of acidified water			
2.4	16.10 (0.06)	576424 (0.13)	12.49 (0.13)

2.5	16.23 (0.04)*	578656 (0.23)	12.54 (0.23)
2.6	16.09 (0.04)	574543 (0.12)*	12.45 (0.12)*
DPR (%)	0.48	0.36	0.36
F_{calc}/F_{crit}	63.35	2.68	2.68

*p<0.01

TABLE 5: CHROMATOGRAPHIC PARAMETERS IN THE ROBUSTNESS TESTS OF THE HPLC METHOD FOR MYSINOIC ACID B (MAB) ASSAY IN *R. FERRUGINEA* STEM BARKS EXTRACTIVE SOLUTION.

Parameter	Average (% CV_{intra})		
	Rt	Area	Assay (mg/g)
Mobile phase flow			
0.8 mL min^{-1}	16.64 (0.03)*	708613 (0.96)	8.34 (0.98)
0.9 mL min^{-1}	15.79 (0.03)*	704757 (0.20)	8.30 (0.23)
1.0 mL min^{-1}	14.95 (0.06)*	663403 (0.37)*	7.85 (0.03)*
DPR (%)	5.37	3.62	3.34
F_{calc}/F_{crit}	9360.45	16.84	16.84
Oventemperature			
34 ºC	15.67 (0.09)	703195 (0.09)	8.28 (0.11)
35 ºC	15.79 (0.03)	704757 (0.20)	8.30 (0.23)
36 ºC	15.62 (0.05)*	711238 (0.21)*	8.37 (0.22)*
DPR (%)	0.52	0.21	0.56
F_{calc}/F_{crit}	46.35	4.86	4.86
pH of acidified water			
2.4	15.66 (0.06)	700666 (0.18)	8.25 (0.22)
2.5	15.79 (0.03)*	704757 (0.20)	8.30 (0.23)
2.6	15.644 (0.04)	702472 (0.11)	8.27 (0.12)
DPR (%)	0.50	0.29	0.27
F_{calc}/F_{crit}	71.91	1.11	1.11

*p<0.01

TABLE 6: CHROMATOGRAPHIC PARAMETERS IN THE ROBUSTNESS TESTS OF THE HPLC METHOD FOR MYSINOIC ACID A (MAA) ASSAY IN *R. FERRUGINEA* FRUITS EXTRACTIVE SOLUTION.

Parameter	**Average (% CV_{intra})**		
	Rt	**Area**	**Assay (mg/g)**
Mobile phase flow			
0.8 mL min^{-1}	17.09 (0,02)*	3333136 (0.87)	79.15 (0.87)
0.9 mL min^{-1}	16.23 (0.04)*	3335657 (0.54)	79.21 (0.54)
1.0 mL min^{-1}	15.38 (0.02)*	3318014 (0.15)	78.78 (0.15)
DPR (%)	5.29	0.29	0.29
F_{calc}/F_{crit}	19786.4	0.13	0.13
Oventemperature			
34 ºC	16.12 (0.04)*	3427112 (0.40)	81.38 (0.40)
35 ºC	16.23 (0.04)*	3335657 (0.54)*	79.21 (0.54)*
36 ºC	16.05 (0.04)*	3455230 (0.27)	82.05 (0.27)
DPR (%)	0.57	1.84	1.84
F_{calc}/F_{crit}	120.16	11.32	11.32
pH of acidified water			
2.4	16.12 (0.04)*	3372613 (0.42)	80.09 (0.42)
2.5	16.23 (0.04)*	3335657 (0.54)	79.21 (0.54)
2.6	16.07 (0.01)*	3443144 (0.42)*	81.77 (0.41)*
DPR (%)	0.52	1.61	1.62
F_{calc}/F_{crit}	149.89	7.18	7.18

*p<0.01

Fig. 2 shows the stem barks extract sample chromatograms, before (**Fig. 2a**) and after the stress degradation tests. In the acidic condition used, the appearance of small, supplementary peaks were observed in the polar region of the chromatogram (**Fig. 2b**, peaks I, II, III and IV), as well as a decrease in the markers peaks. The UV absorption profiles of these peaks were similar to

found for isolated MAA and MAB [20], indicating its possible degradation products by hydrolysis, what requires further studies for structural elucidation. The degradation in oxidative media (**Fig. 2c**), after UV (**Fig. 2d)** and visible exposure (**Fig. 2e**) provided less percentages of markers degradation and no significant alteration in the chromatographic profile of the *R. ferruginea* stem barks extract.

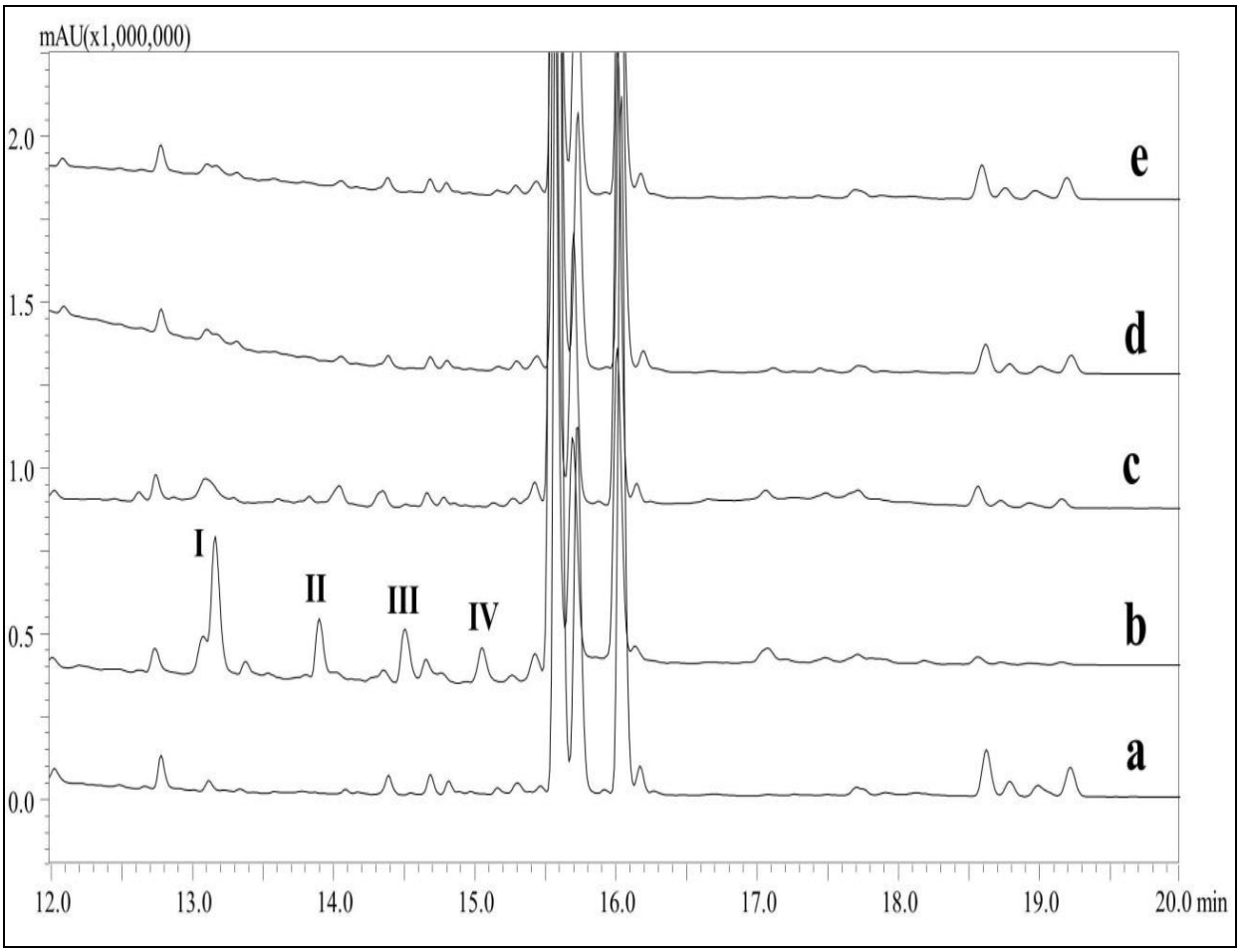

FIG. 2: CHROMATOGRAPHIC PROFILES AT 270 nm OF *R. FERRUGINEA* STEM BARKS EXTRACT AT 1.0 mg mL^{-1}: (a)SUBMITTED TO STRESS TESTS FRESH SAMPLE SOLUTION; (b) AFTER STRESS WITH 1 mol L^{-1}HCl 24 h; (c) AFTER STRESS WITH 30% H_2O_2 4 h; (d) AFTER UVA EXPOSURE AT 400 Whm^2; (e) AFTER VISIBLE LIGHT EXPOSURE AT 2.4 mi lux h^{-1}.

Under different stress degradation conditions, *R. ferruginea* fruits extract showed in acidic conditions, the formation of three major degradation products, more polar than MAA (**Fig.3b**). The corresponding peaks I', II' and III' had the same UV profile and retention times than peaks II and IV, detected in stem barks extract sample degraded in the same conditions, suggesting MAA degradation products. In spite of intense percentage of MAA degradation under oxidative condition **(Table 3**), were observed only small supplementary peaks in the chromatogram (**Fig. 3c**). The effect of UVA exposure resulted in a decrease of MAA and other more polar peaks of chromatogram. At this condition, the impurities corresponding to groups of peaks IV' and V' were observed (**Fig. 3d**), with UV profiles similar to benzoic acid derivatives. **Fig. 3e** presents the chromatogram of fruits extract after visible light exposure, where is observed a significant decrease in MAA (**Table 3**) however, few supplementary peaks, as the presence of the same impurity named peak V' that was also apparent in UVA exposed sample. The developed HPLC method presents a more complete profile of the extracts, compared with previously developed isocratic method [22], allowing the separation of the degradation products after subjecting the extracts to stress conditions.

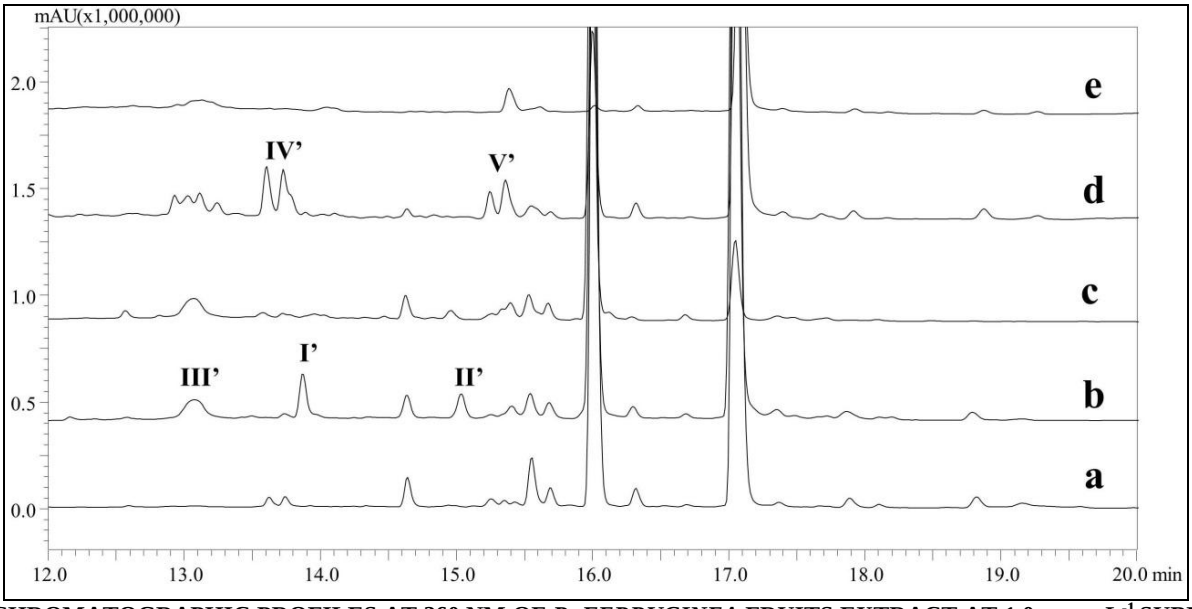

FIG. 3: CHROMATOGRAPHIC PROFILES AT 260 NM OF *R. FERRUGINEA* FRUITS EXTRACT AT 1.0 mg mL^{-1} SUBMITTED TO STRESS TESTS: (a) FRESH SAMPLE SOLUTION; (b) AFTER STRESS WITH HCl 1 mol L^{-1} 24 h; (c) AFTER STRESS WITH 30% H_2O_2 4 h; (d) AFTER UVA EXPOSURE AT 400 Whm^2; (e) AFTER VISIBLE LIGHT EXPOSURE AT 2.4 mi lux h^{-1}.

Assay of MAA and MAB in extracts and nanoemulsions:

The assays of MAA and MAB in *R. ferruginea* stem barks extract were found to be 4.91 % and 4.38 %, respectively. In fruits extract the assay of MAA was 14.98 %. The method developed was also applied to ensure the analytical identification and quantification of MAA and MAB in nanoemulsions containing *R. ferruginea* stem barks and fruits extract. The assays of MAA and MAB in the stem. The assays of MAA and MAB in the stem barks extract nanoemulsions were found to be 0.006 % (93.8% of theoretical value) and 0.0055 % (96.5% of theoretical value), respectively. In the fruits extract nanoemulsions, the assay of MAA was 0.071 % (104.4% of theoretical value).

Preliminary stability study of nanoemulsions:

When evaluated through stability at room temperature storage (25ºC), NSB showed a significant and gradual degradation of the markers MAA and MAB until 6 months. At 40 °C storage, both markers suffered degradation, but it was more pronounced for MAA, as presented in **Fig. 4.** The NF formulation showed a different behavior, as observed at **Fig. 4**. At 2 months of storage at room temperature (25°C), no significant influence on MAA assay was observed. When stored for 2 months at 40 °C, the NF showed a gradual increase on MAA assay, probably due the evaporation of formulation.

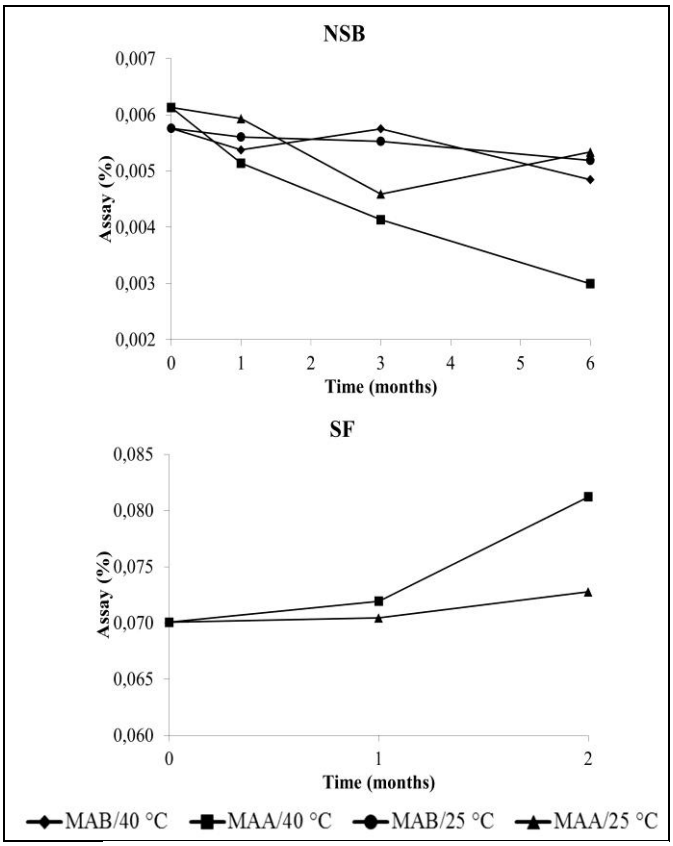

FIG. 4: ASSAY OF MAA AND MAB AFTER ACCELERATED STUDIES WITH NANOEMULSIONS CONTAINING *R. FERRUGINEA* STEM BARKS (NSB) AND FRUITS (NF) EXTRACTS.

NSB subjected to room temperature storage did not show significant changes in chromatograms **(Fig. 5a)**. For NSB storage at 40 °C (**Fig. 5b**) was

noticed the presence of some impurities, probably due to the formulation components degradation. At 16.2 min, after 3 months at 40 ºC it was observed an impurity named VI' with similar UV profile when compared to MAA, suggesting its degradation product, once MAA peak showed a decrease of intensity. For the chromatographic profiles of NF samples subjected to thermal stress at room temperature and at 40 ºC (**Fig. 5c** and **d**), no changes in MAA peak were observed and triglyceride peak showed an intense decrease after 2 months of storage under both conditions. However, no degradation products were detected for these samples, probably due to the lack of chromophoric groups in the degradation products formed during this study.

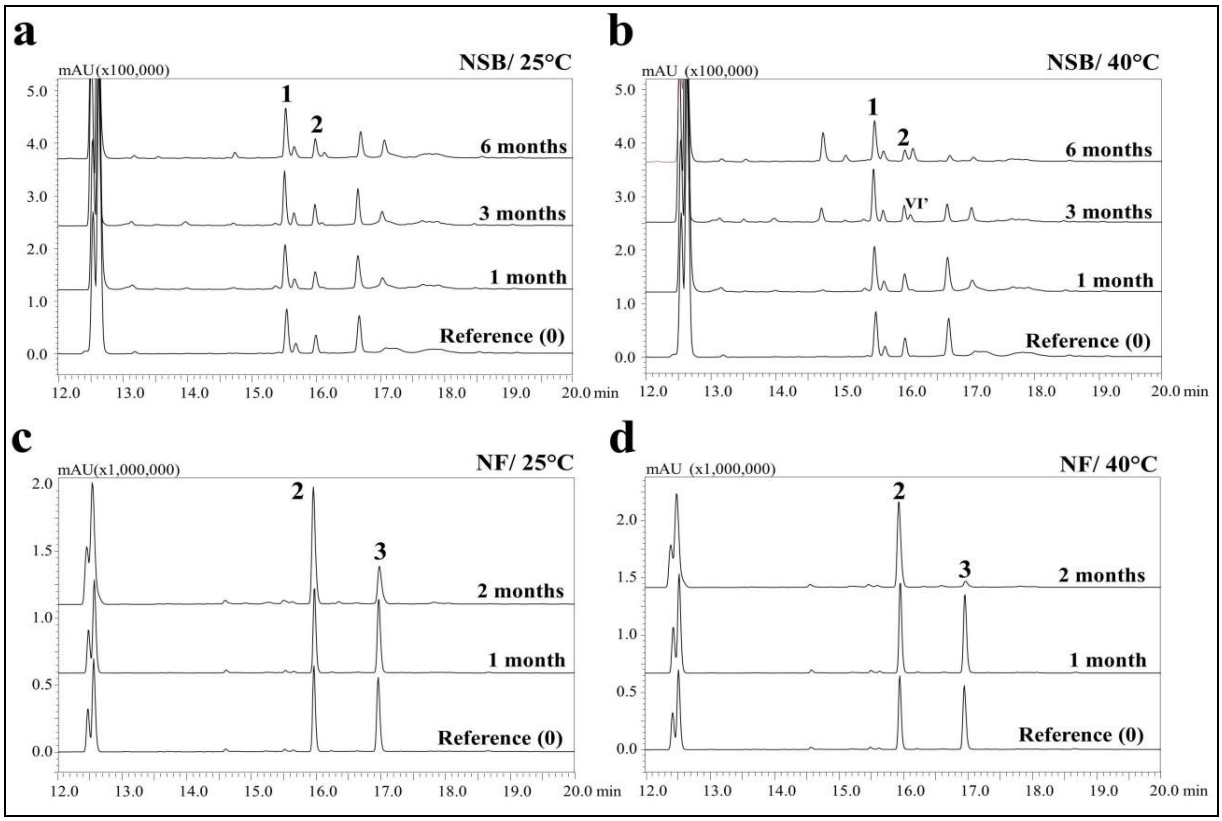

FIG. 5: CHROMATOGRAMS SHOWING SEPARATION BEHAVIOUR OF MAB (1) AND MAA (2) IN NANOEMULSIONCONTAINING *R. FERRUGINEA* STEM BARKS EXTRACT SUBJECTED TO ROOM TEMPERATURE (a) AND 40 °C (b) AND FOR 0, 1, 3 AND 6 MONTHS AT 270 nm AND IN NANOEMULSION CONTAINING *R. FERRUGINEA* FRUITS EXTRACT SUBJECTED TO ROOM TEMPERATURE (C) AND 40 °C (D) 0, 1 AND 2 MONTHS AT 260 nm

Under almost stress conditions, the MAA presented higher lability than MAB, both, in stem barks and fruits extracts, but the latter showed be less stable than the former extract, probably due to their different composition and higher concentration of MAA. The functional groups with labile hydrogens present in MAA and MAB chemical structures are susceptible to oxidation reactions, as well as light stress conditions can induce photo oxidation by free radical mechanism [18]. Regardless of degradation products, there was no interference in retention time of markers, since peak purity remained > 99.99 % for both compounds in all tested conditions, showing the selectivity and stability indicative capability of the developed method. The method applied to stability studies of nanoemulsions allowed the quantification of MAA and MAB without interference. The analytical method allows application of support future improvements in the nanomulsion formulations which were not stable at high temperatures, a challenge to herbal derivatives in nanoemulsions, since, in general, studies are conducted at low temperatures [23].

CONCLUSIONS: A validated stability indicating HPLC-DAD method for the determination of MAA and MAB in *R. ferruginea* extracts and nanoemulsions was developed. The gradient method was specific, linear, sensitive, accurate, robust and did not suffered influence of degradation products on MAA and MAB peaks.

The method can be useful during stability studies of *R. ferruginea* extracts and its related nanoemulsions.

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