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Article

Stability of hydroalcoholic extracts of two species of *guaco; Mikania glomerata* SPRENG. and *Mikania laevigata* SCHULTZ. (Asteraceae), by UHPLC-MS

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It is important to study the stability of plant extracts used as active ingredients in phytotherapic medicine, as degradation of the active principles directly affects the efficacy and safety of these products. Therefore, a stability study of the hydroalcoholic extract of the species: *Mikania glomerata* and *Mikania laevigata* was conducted in order to determine the speed of degradation and shelf life of these extracts, which are incorporated in cough syrup in Brazil. Leaves of both species were dried in an oven or by lyophilization (freeze-dried). Hydroalcoholic extracts underwent both accelerated stability study of six months and long-term stability study for 12 months. Samples were stored at different temperatures and every three months were analysed by ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) to monitor their chemical profile, quantifying coumarin and chlorogenic acid. For all conditions of the study, a reduction of the content of the chemical marker of this species, coumarin, greater than 5% was observed, so a shelf life of two years cannot be assigned to the hydroalcoholic extracts of these species as observed in commercial extracts.

Keywords: *Mikania glomerata* Spreng.. *Mikania laevigata* Sch. Bip. ex Baker.. *Guaco*. Ultra-high performance liquid chromatography-mass spectrometry. Stability test. Coumarin. Chlorogenic acid.

INTRODUCTION

In Brazil, two species of Mikania, popularly known as guaco are easily available and widely used in popular medicine in extemporary preparations such as tea and homemade syrups for the treatment of diseases of the airways, due to their bronchodilator effect (Silva *et al*., 2008). *Mikania glomerata* Spreng. and *Mikania laevigata* Sch. Bip. ex Baker extracts are also found in industrialized syrups, which therefore need to have a longer and clearly defined shelf life (Gasparetto *et al*., 2010). To guarantee the efficacy, safety and quality of herbal medicines is often a challenge (Pinto *et al*., 2002). By virtue of the therapeutic properties attributed to guaco, syrup and oral solution of *M. glomerata* were included in the reference list of medicines for primary health care in 2007 (Brasil, 2007). Therefore, herbal medicines based on guaco are used extensively in the public health system and by the general population (ANVISA, 2008; Brasil, 2006).

A norm, published by the Brazilian government agency classified 36 medicinal plants as safe and effective and only the manufacturing process and quality control need be detailed for their registration. These plants have streamlined registration, including *M. glomerata*, enabling their industrial production (ANVISA, 2008). Another resolution establishes the minimum requirements for the registration of herbal medicine and lists the documents required for registration (ANVISA, 2010), including results of accelerated stability study or the long-term stability studies, according to Government standards (Brasil, 2005).

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Stability tests aim to establish the shelf life, period of use in packaging and storage conditions of a specific drug. In general, the shelf life of a product is determined by the long-term stability study of 12 months, but for purposes of registration, a 6 month-study of accelerated stability is accepted (Brasil, 2005). Although this test is not mandatory for active components (such as guaco extracts used in syrups), commercial guaco extracts (tinctures and liquid extracts of *M. glomerata* and *M. laevigata*) were acquired with expiration dates indicating shelf lives of over 2 years, possibly without the evaluation of their stability.

Given the lack of studies evaluating the stability of extracts of *M. glomerata* and *M. laevigata*, prepared according to pharmacopoeia standards, the objective of this work was to study the stability of hydroalcoholic extracts of *M. glomerata* and *M. laevigata* in accordance with the provisions of the Guide for conducting Stability Studies Resolution (Brasil, 2005) accompanying the content of their official chemical marker, coumarin.

MATERIAL AND METHODS

Material

Specimens of *M. glomerata* and *M. laevigata* growing in the experimental field of the Department of Plant Biology at the Institute of Biology, UNICAMP, and their exsiccates are deposited in the herbarium of the Universidade Estadual de Campinas under registration UEC No. 102047 for *M. glomerata* and No. 102046 for *M. laevigata*.

Leaves of both species were collected in the morning and divided into two groups, the first being subjected to drying in an oven with air circulation at 40 ºC for 50 hours. The second group was frozen and lyophilized for 50 hours. The dried leaves of the two groups were ground (0.84 mm particle size). The extracts were prepared following the First Brazilian Pharmacopoeia (Brasil, 1929) using a proportion of 200 g of the powdered leaves to 1000 mL of 67% (v/v) alcohol solution (Ecibra, Brazil). After extraction, an aliquot of 10 mL was dried at 105 ºC until constant weight to determine the solids content.

Chromatographic method

For the chromatographic evaluation the validated method (Melo, Sawaya, 2015) was used. An ultra-high performance liquid chromatographer coupled to a triple quadrupole mass spectrometer, Acquity UPLC-TQD (Micromass, Waters, Manchester, England) was used with a Waters Acquity BEH C18 column (2.1 mm x 50 mm, 1.7 µm particles) and oven temperature of 30 ºC. Elution was performed at a flow of 200 µL/min with solvent A - purified water (Milli-Q) with 0.1% formic acid and solvent B - Acetonitrile HPLC grade (JT Baker, PA, USA). The elution gradient started with 10% solvent B increasing to 25% B in 4 minutes, then 100% B in 8 min; 100% B was maintained until 8.5 min, the returning to the initial conditions and stabilizing up to 10 min. MS detection was performed in positive and negative ion mode with electrospray ionization under the following conditions: 3000 V capillary ± cone ± 35 V, source temperature 150 ºC and desolvation temperature of 300 ºC.

Before the injection, the extracts were diluted with purified water (Milli-Q) at a ratio of 1 part to 2 parts of the extract and 2 µL of each sample was injected. The concentration of coumarin and chlorogenic acid in the extracts was quantified using a calibration curve of coumarin (Sigma-Aldrich) and chlorogenic acid (Sigma-Aldrich) in 70% ethanol solutions and blank plant matrix (Melo, Sawaya, 2015). Chlorogenic acid was analysed in the negative ion mode and coumarin in positive ion mode.

Results were analysed using GraphPad Prism, version 5.00 for Windows (Microsoft), GraphPad Software, San Diego, California, USA, expressed as mean ± standard error and were compared by Tukey test at 5% probability ($p<0.05$).

Stability Study

The stability study (Brasil, 2005) of the hydroalcoholic extracts was carried out in glass bottles. The extracts were stored in clear glass bottles for 12 months at ambient temperature of 22 ºC in the presence and absence of light. For the 6-month accelerated study, samples were kept at 40 ± 2 ºC without controlled humidity, as determined by the guide for conducting stability studies. For the long-term study, samples were kept at 30 ± 2 ºC for 12 months without controlled humidity. After the storage period, the initial volume of the extracts was supplemented with a solution of 70% ethanol and then diluted in purified water (Milli-Q) at a ratio of 1: 2 extract: water prior to analysis by UHPLC- MS.

In addition to the extracts prepared in our lab, samples of commercial extracts obtained from a

distributor (guaco tincture and guaco fluid extract) were also subjected to an accelerated stability study. The concentration of coumarin and chlorogenic acid was accompanied during the stability study.

RESULTS AND DISCUSSION

The drying method only slightly affected the solid residue, as reported previously: 1.3% (w/v) for oven dried *M. glomerata* compared to 1.4% (w/v) for freeze-dried *M. glomerata*, and 1.2% (w/v) for oven dried *M. laevigata* to 1.3% (w/v) for freeze-dried *M. laevigata*. The initial chromatographic analysis also showed a similar chromatographic profile for each species, regardless of the form of drying. However, a marked difference in the concentration of the chemical marker, coumarin, was observed between species. Another compound, chlorogenic acid, was lower in the oven dried samples indicating that oven drying resulted in the loss of some constituents (Melo, Sawaya, 2015).

The initial concentration of chlorogenic acid in oven dried *M. glomerata* extract was 1348 μg mL^{-1} and 1934 μg.mL^{-1} for the freeze-dried extract. Coumarin values for the *M. glomerata* extracts were below the limit of quantitation (LOQ = 3.3 μg mL^{-1}) and therefore could not be analysed in this study. For the extract of oven dried *M. laevigata*, a concentration of 775.1 μg mL^{-1} coumarin was found and 1131 μg mL^{-1} for the extract of freeze-dried leaves. The concentration of chlorogenic acid in extracts of *M. laevigata* for both drying processes was below the limit of quantitation (LOQ = 20.4 μg mL^{-1}) and therefore could not be analysed in this study. Due to differences in the composition of the extracts of both species, coumarin was used as the marker for *M. laevigata* in the stability study and chlorogenic acid in *M. glomerata*.

The initial content of coumarin and chlorogenic acid in commercial samples of fluid extract and tincture of guaco was also quantified: 358.2 μg mL^{-1} of coumarin and 159.0 μg mL^{-1} of chlorogenic acid in the fluid extract and 232.3 μg mL^{-1} of coumarin and 143.7 μg mL^{-1} of chlorogenic acid in the tincture of guaco. These results were surprisingly different from those of our extracts, indicating that the extracts were possibly obtained from a mixture of leaves from both species or using the stems, because this part of the plant presents coumarin in both species, although in different concentration (Costa et. al., 2018). These extracts were also submitted to the accelerated and long-term stability studies.

Only chlorogenic acid was quantified in the extracts of *M. glomerata*, as the coumarin concentration was below the LOQ. During the long-term stability study at 30 ºC (Figure 1), the content of chlorogenic acid was not significantly different after the first 3 months of the study for extracts of oven dried *M. glomerata*; but was significantly different after this period. The same occurred for the accelerated stability study of extracts of oven dried *M. glomerata* (40 ºC). For the freeze-dried samples, there was a significant difference in the chlorogenic acid content already within the first 3 months of the long-term stability study (30 ºC); the same did not happen for the accelerated stability study. Comparing the accelerated and long-term stability studies, in 3 months, oven dried samples showed no significant difference in the content of chlorogenic acid but the freeze-dried samples did. The initially higher concentration chlorogenic acid in extracts of freeze-dried samples was equal to that of oven dried samples after 6 months at 40 ºC whereas after 12 months at 30 ºC the content of chlorogenic acid was still significantly higher in the freeze-dried extracts. Therefore, the higher the temperature at which leaves are dried significantly affected the content of chlorogenic acid in the *M. glomerata* extracts.

For the long-term stability study at room temperature (22 ºC) in the presence or absence of light (Figure 2) the content of chlorogenic acid was not significantly different after 3 months for extracts of oven dried *M. glomerata* ($p<0.05$), but decreased significantly afterwards. However, for the extracts of freeze-dried *M. glomerata*, at 22 ºC with and without light, there was a significant decrease in the content of chlorogenic acid from 3 months onward. No significant differences were observed between samples kept in the light or dark. As the concentration of all samples dropped over time, after 12 months there were no significant differences between the extracts of oven dried and freeze-dried *M. glomerata* leaves. The average concentration of chlorogenic acid in extracts of oven dried and freeze dried leaves *M. glomerata* leaves after 12 months at 30 ºC were 510.4 μg mL^{-1} and 577.8 μg mL^{-1} respectively; after 12 months at 22 ºC with light were 468.5 μg mL^{-1} and 522.0 μg mL^{-1} respectively and after 12 months at 22 ºC without light were 496.2 μg mL^{-1} and 595.9 μg mL^{-1} respectively. These results indicate that the time in storage affected the concentration more than the temperature or incidence of light, with the freeze-dried leaf extracts generally presenting a slightly higher concentration.

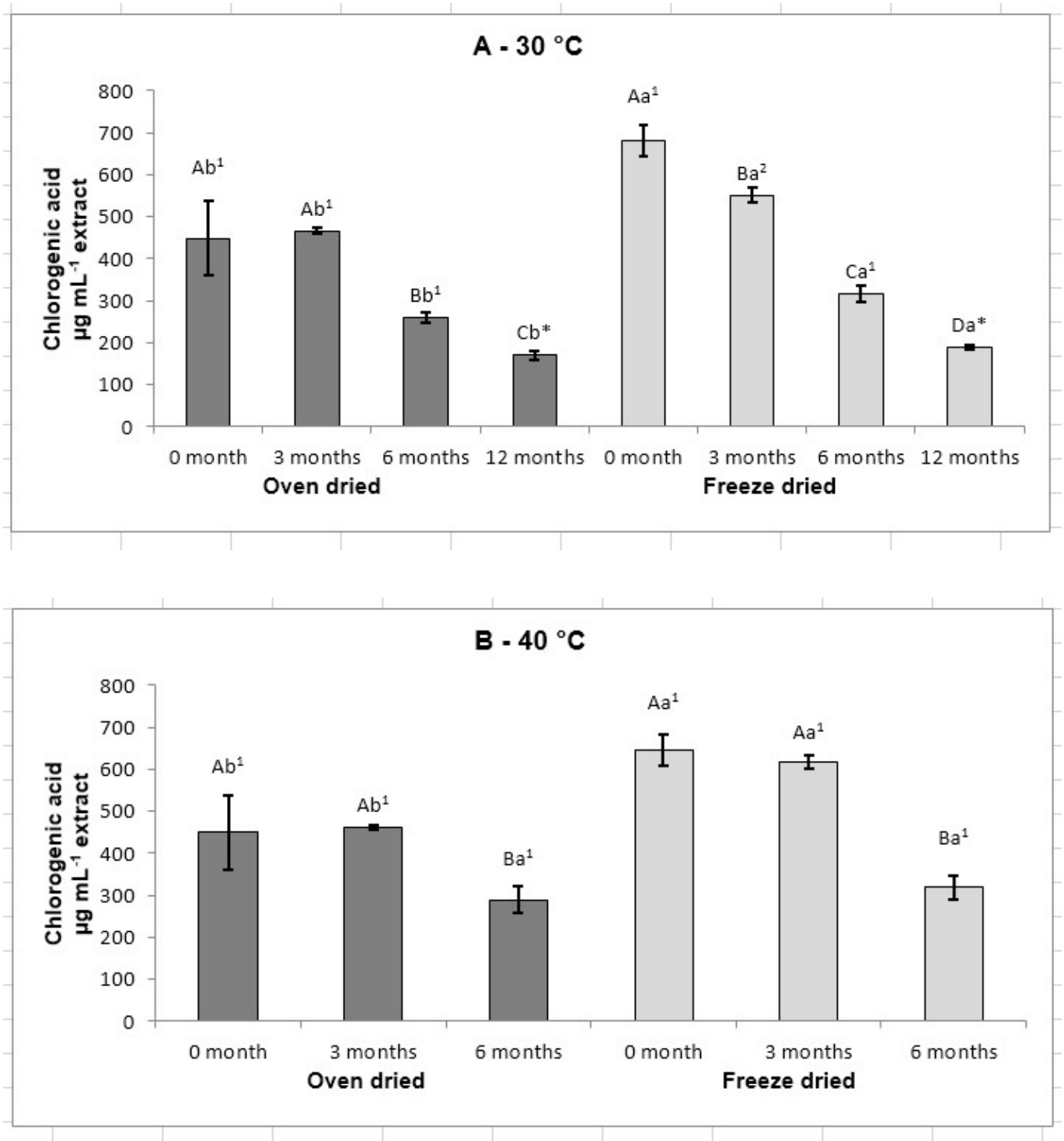

FIGURE 1 - Variation of the content of chlorogenic acid in extracts of oven dried and freeze-dried *M. glomerata* leaves. (A) long-term stability study at 30 ºC and (B) accelerated stability study at 40 ºC. (*) Indicates no data collected for this group. Capital letters indicate statistically significant ($p<0.05$) differences within the same drying method (0, 3, 6 and 12 months). Lowercase letters indicate statistically significant ($p<0.05$) differences between drying methods at the same time. Numerals indicate statistically significant ($p<0.05$) differences between storage temperatures (30 ºC and 40 ºC). Experiment performed in triplicate. Error bars indicate standard deviation.

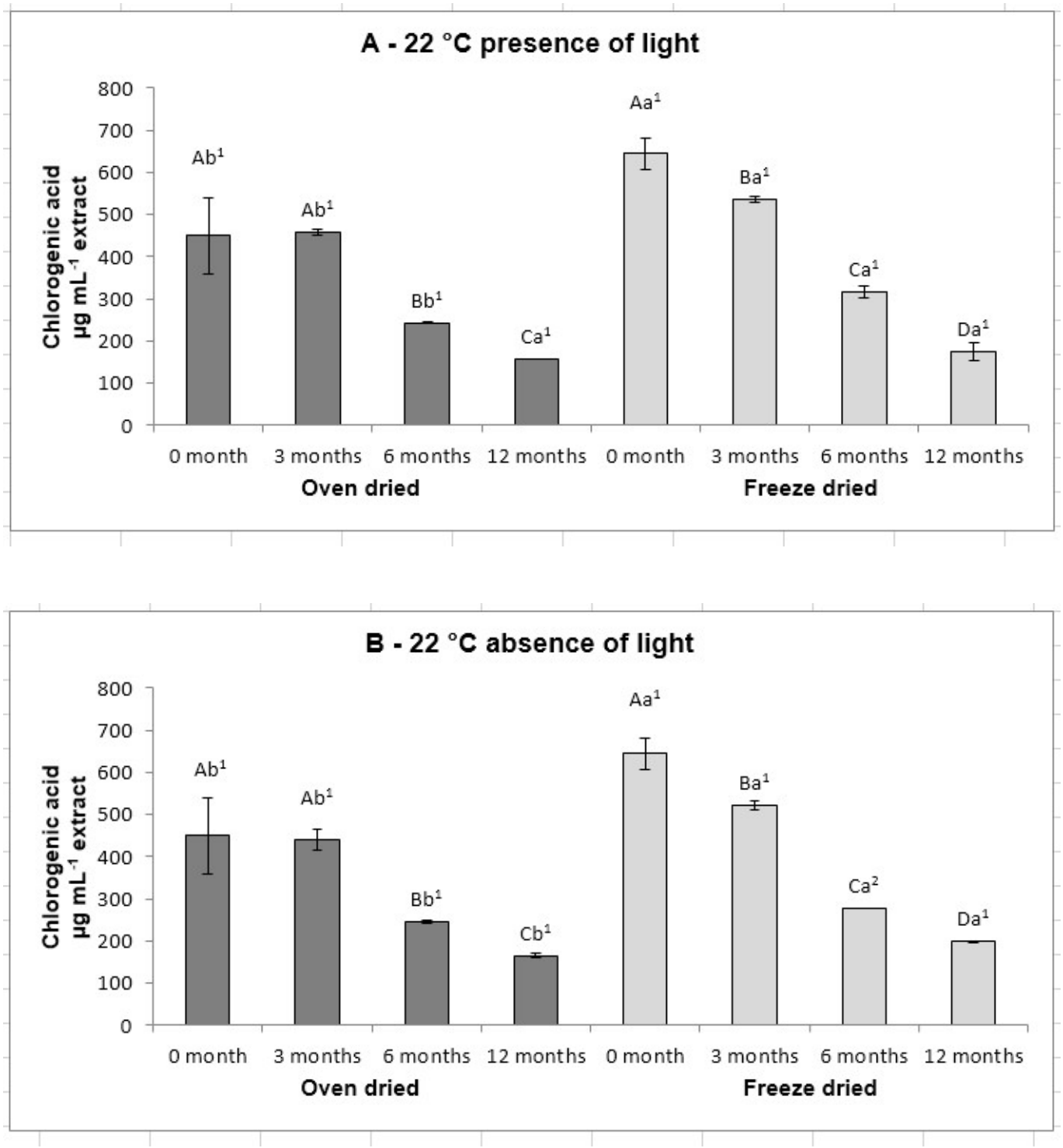

FIGURE 2 - Variation of the content of chlorogenic acid in extracts of oven dried and freeze-dried *M. glomerata* leaves. Long term stability study at 22 ºC in the presence of light (A) and absence of light (B). Capital letters indicate statistically significant ($p<0.05$) differences within the same drying method (0, 3, 6 and 12 months). Lowercase letters indicate statistically significant ($p<0.05$) differences between drying methods at the same time. Numerals indicate statistically significant ($p<0.05$) differences between storage with and without light. Experiment performed in triplicate. Error bars indicate standard deviation.

Coumarin was quantified in the extracts of *M. laevigata*, as the chlorogenic acid concentration was below the LOQ. During the long-term stability study at 30 ºC and accelerated study at 40 ºC (Figure 3), the content of coumarin was not significantly different after the first 3 months of the study for oven dried extracts of *M. laevigata*; being significantly different only after this period. For the extracts of freeze-dried samples, there was a significant decrease in the coumarin content from the first 3 months onward. This result is similar to that observed for *M. glomerata* extracts (Figures 1 and 2). The concentration of coumarin was initially higher in freeze-dried extracts and remained significantly higher throughout this study.

The results of the long-term stability study (22 ºC), in the presence and absence of light, indicate a significant decrease ($p<0.05$) in the coumarin concentration along the year (0, 3, 6 and 12 months) for the extracts of oven dried *M. laevigata* leaves. The same happened for the freeze-dried leaf extract, which initially had higher coumarin content and remained significantly higher throughout the study. No significant differences were observed due to the presence of light (Figure 4).

The variation of the chlorogenic acid and coumarin contents in commercial samples of fluid extract and tincture of guaco submitted to accelerated stability study (40 ºC) is shown in Figure 5. The initial concentration of both samples was not significantly different. In both cases there was a significant increase in the coumarin and chlorogenic acid contents after 3 months, which is difficult to explain. After 6 months these concentrations dropped below the initial concentrations. This uncommon result was also reported by Amaral *et al*., 2009 who attributed the increase in coumarin to solvent loss by evaporation or the balance of cis-trans isomers of o-coumaric acid in function of the temperature, promoting the cyclization of o-coumaric acid into coumarin. However, this does not explain why a significant increase was observed only in commercial extracts which, if prepared according to the Pharmacopoeia, should not have any antioxidants or other compounds added.

The percentages of coumarin content reduction is shown in Table I and of chlorogenic acid in Table II. According to Brazilian legislation (Brasil, 2005) a proposed shelf life of 24 months is only approved if the accelerated stability study or long-term study show less than 5.0% decrease in the active contents. Should this decrease be between 5.1% and 10.0% in the accelerated stability study, the proposed shelf life will be reduced by half, to 12 months. There was a decrease of far more than the 5 or 10%, for coumarin (the chemical marker) as well as for chlorogenic after 6 months, for the extracts prepared in our laboratory and for the commercial samples. Therefore, the shelf life of the *M. laevigata* extracts and commercial extracts, considering the decrease in coumarin concentration, should be less than 6 months, possibly only 3 months.

The accelerated and long-term degradation studies of *M. laevigata* extracts, as well as of commercial samples indicate that the shelf life of the extracts should not be higher than 3 months, considering the decrease in coumarin concentration. It can also be noted that the storage temperature and light incidence did not strongly affect this decrease, as the percentage of reduction of coumarin after 12 months at 30 *ºC* or at 22 *ºC* with or without light were similar. Although chlorogenic acid is not an official chemical marker for *M. glomerata*, the decrease in its concentrations followed the same trend as observed for coumarin (Table II). It is important to study the stability of plant extracts used as active ingredients in phytotherapic medicine, as degradation of the active principles directly affects the efficacy and safety of these products.

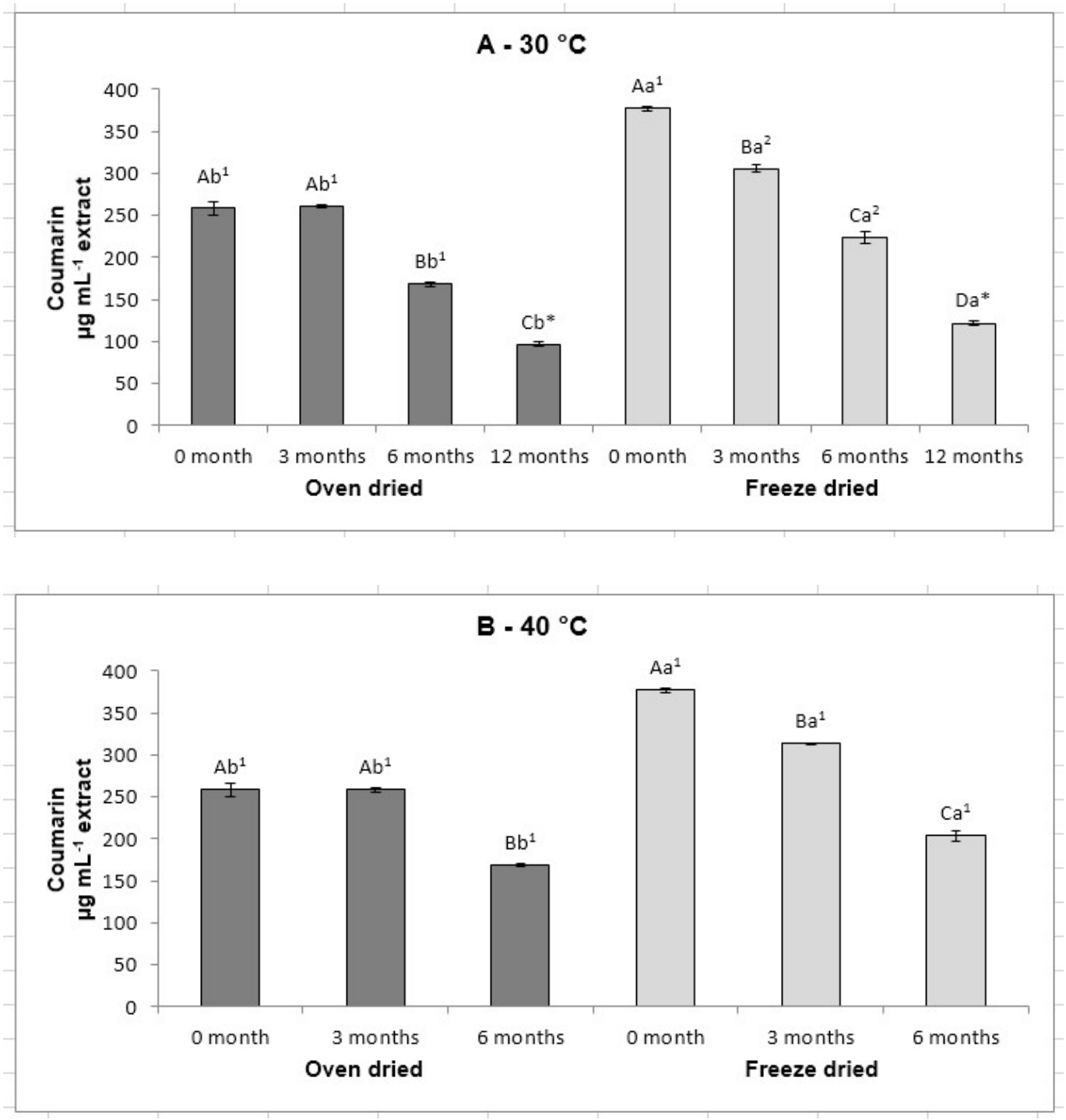

FIGURE 3 - Variation of the content of coumarin in extracts of oven dried and freeze-dried *M. laevigata* leaves. (A) long-term stability study at 30 ºC and (B) accelerated stability study at 40 ºC. (*) Indicates no data collected for this group. Capital letters indicate statistically significant ($p<0.05$) differences within the same drying method (0, 3, 6 and 12 months). Lowercase letters indicate statistically significant ($p<0.05$) differences between drying methods at the same time. Numerals indicate statistically significant ($p<0.05$) differences between storage temperatures (30 ºC and 40 ºC). Experiment performed in triplicate. Error bars indicate standard deviation.

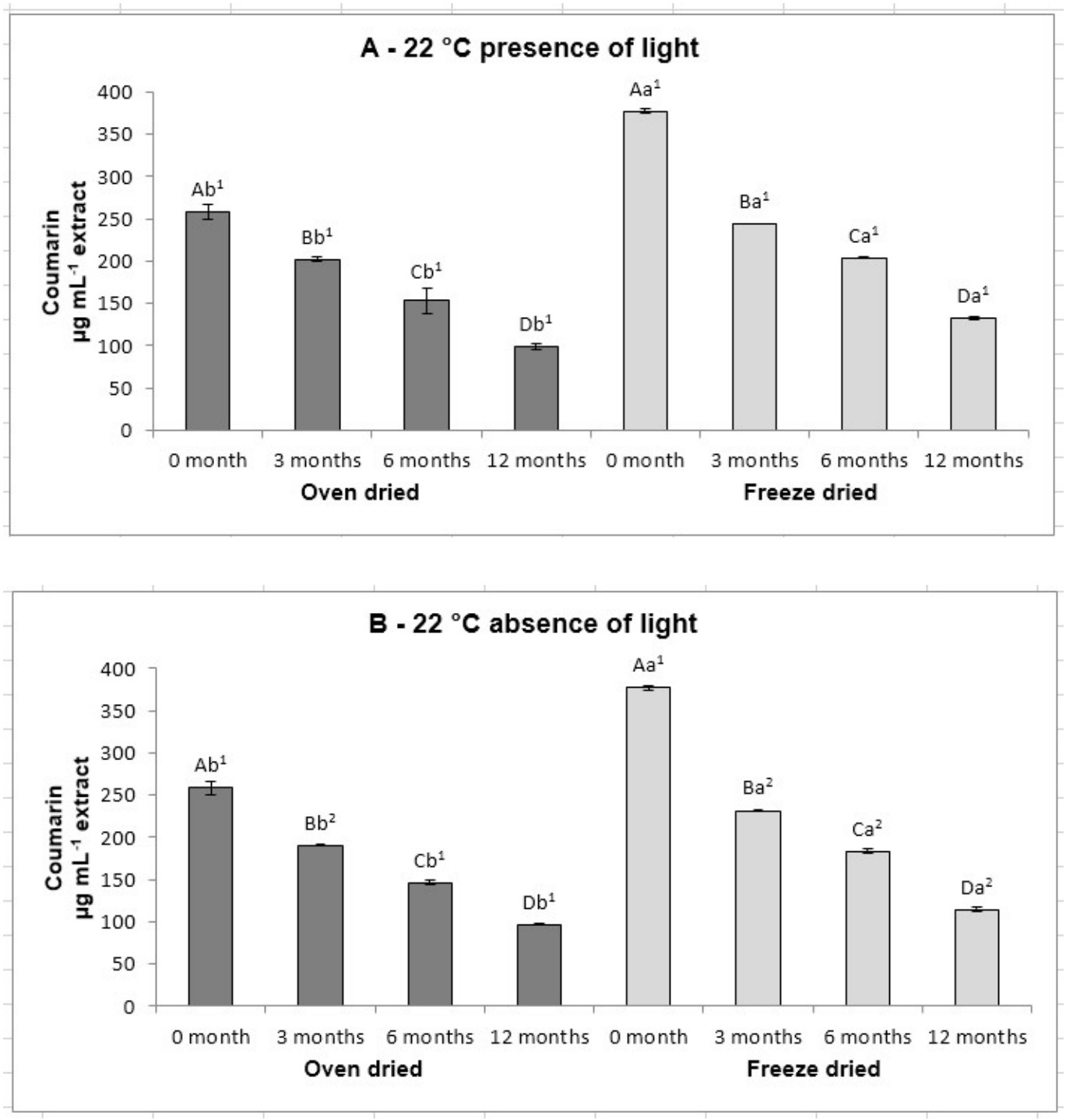

FIGURE 4 - Variation of the content of coumarin in extracts of oven dried and freeze-dried *M. laevigata* leaves. Long term stability study at 22 ºC in the presence of light (A) and absence of light (B). Capital letters indicate statistically significant ($p<0.05$) differences within the same drying method (0, 3, 6 and 12 months). Lowercase letters indicate statistically significant ($p<0.05$) differences between drying methods at the same time. Numerals indicate statistically significant ($p<0.05$) differences between storage with and without light. Experiment performed in triplicate. Error bars indicate standard deviation.

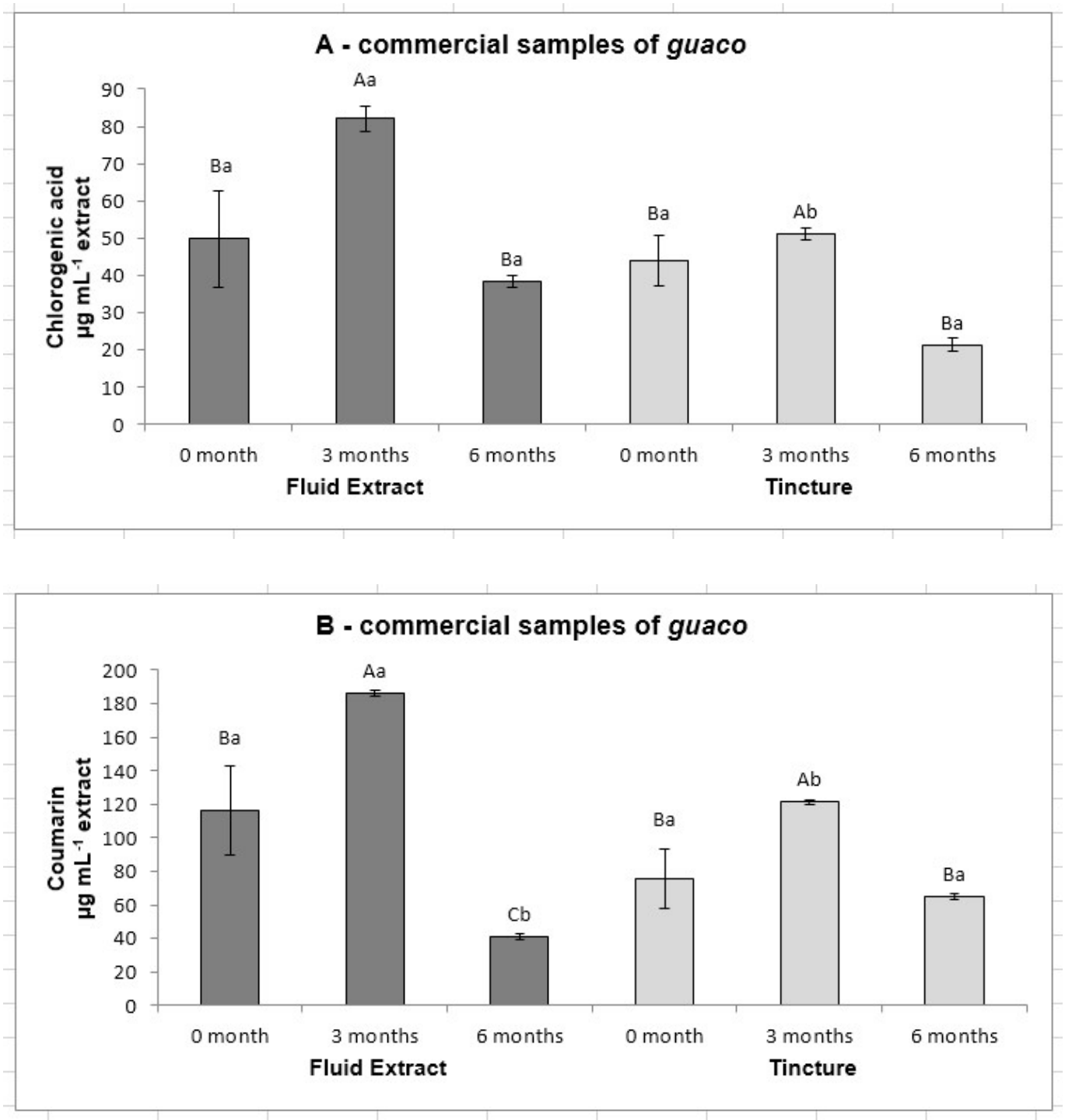

FIGURE 5 - Variation of the content of chlorogenic acid (A) and coumarin (B) in commercial samples of *guaco* (Fluid Extract and Tincture of *guaco*) subjected to accelerated stability study (40 ºC). Capital letters indicate statistically significant ($p<0.05$) differences for the same sample lowercase letters indicate statistically significant ($p<0.05$) differences between samples. Experiment performed in triplicate. Error bars indicate standard deviation.

TABLE I - Percentage of coumarin reduction for the evaluated extracts

Sample	% after 3 months	% after 6 months	% after 12 months
Long term stability study (30 °C)			
MLO	+ 1	- 35	- 62
MLL	- 19	- 41	- 68
Accelerated stability study (40 °C)			
FE	+ 60	- 65	N
TG	+ 61	- 14	N
MLO	- 19	- 35	N
MLL	- 34	- 46	N
Stability study at room temperature with light (22 °C)			
MLO	- 22	- 41	- 62
MLL	- 35	- 46	- 65
Stability study at room temperature without light (22 °C)			
MLO	- 26	- 43	- 62
MLL	- 39	- 51	- 70

Abbreviations: MLO: *M. laevigata* oven dried; MLL *M. laevigata* lyophilized; FE: fluid extract of *guaco*; TG: tincture of *guaco*; symbol (+): Increase in the content; symbol (-): Decrease in the content; N: Not evaluated.

TABLE II - Percentage of chlorogenic acid reduction for the evaluated extracts

Sample	% after 3 months	% after 6 months	% after 12 months
Long term stability study (30 °C)			
MGO	+ 4	- 42	- 62
MGL	- 12	- 48	- 70
Accelerated stability study (40 °C)			
FE	+ 53	- 18	N
TG	+ 13	- 41	N
MGO	+ 3	- 36	N

(continuing)

TABLE II - Percentage of chlorogenic acid reduction for the evaluated extracts

Sample	% after 3 months	% after 6 months	% after 12 months
MGL	- 4	- 50	N
Stability study at room temperature with light (22 °C)			
MGO	+ 2	- 46	- 65
MGL	- 17	- 51	- 73
Stability study at room temperature without light (22 °C)			
MGO	- 2	- 45	- 63
MGL	- 19	- 57	- 69

Abbreviations: MGO: oven dried *M. glomerata*; MGL: freeze dried *Mikania glomerata*; FE: fluid extract of *guaco*; TG: Tincture *guaco*; symbol (+): Increase in the content; symbol (-): Decrease in the content; N: Not evaluated.

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