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Thermal and photo stability of phenolic constituents of an *Achyrocline satureioides* spray-dried powder

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Thermal and the photo stabilities of an *Achyrocline satureioides* powder (SDP40) were evaluated in particular concerning the total polyphenol content as well as the main identified constituents quercetin, luteolin, 3-*O*-methylquercetin and caffeic acid. SDP40 presented good stability for nine months under normal storage conditions of 25 °C temperature and 60% relative humidity (RH). In accelerated term testing, 50 °C temperature and 90% RH and also in stress testing, 80 °C, caffeic acid and a non-identified constituent P3 were the most instable constituents. Luteolin and 3-*O*-methylquercetin were the most stable constituents. Quercetin presented an unusual behavior, improving its concentration after 1 month at 50 °C or 2 days at 80 °C exposition, followed by a decrease in its concentration. The hypothesis that this observation is related to the simultaneous decreasing of a non-identified peak P3 or to the hydrolysis of a non-identified precursor as a quercetin heteroside is being investigated. The SDP40 presented good stability against UV-C light when conditioned in amber or transparent containers, but it suffered degradation when stored in open-dishes. In summary, the total polyphenol content remains within acceptable limits of 10% under normal storage conditions for nine months. However, the LC polyphenol analysis demonstrated that the behavior of individual constituents has still to be enlightened.

1. Introduction

Achyrocline satureioides (Lam) DC, Asteraceae, is a medicinal plant distributed in subtropical South America, used in folk medicine of the South of Brazil, Uruguay, Paraguay and Argentine as anti-inflammatory, antiulcerative and antispasmodic (Simões et al. 1998).

The flavonoids quercetin, luteolin and 3-*O*-methylquercetin have been regarded as the main constituents of the inflorescences ethanol extracts. Several pharmacological activities have been related to the presence of these constituents, like antioxidant, anti-inflammatory, anti-ulcerative, anti-hepatotoxic, antispasmodic, antiplatelet aggregation, vasodilatory and antiviral effects (Husain et al. 1987; Formica and Regelson 1995; Pelzer et al. 1998; Harbone and Smith 2000; Di Carlo et al. 2002; Betega et al. 2004; Polydoro et al. 2004).

Liquid extracts, such as hydroethanolic extracts, are still the most widely used herbal pharmaceutical preparations due to their low cost and easiness to prepare. However, the low stability of herbal liquid preparations, dramatically affected by ethanol content and the lability of the constituents represent the main disadvantage of this type of products (Bilia et al. 2002).

Spray drying is a technique widely employed for producing powders containing some heat sensitive constituents due to the quick solvent evaporation (Broadhead et al. 1992). This characteristic made this technique particularly useful for preparing medicinal herbal powders from the corresponding liquid extracts (Lemos Senna et al. 1997). Spray dried powders from *Achyrocline satureioides* inflorescences have been previously reported being the process and the product deposited as a patent (Bassani et al. 2001). More recently, an *Achyrocline satureioides* spray-dried powder obtained from an extractive solution containing 40% (v/v) ethanol, (SDP40), has been reported as presenting higher anti-inflammatory activity than that obtained from liquid extract prepared with ethanol 80% or hot water as solvent (De Souza et al. 2007).

Thus, although several biological activities have been already demonstrated for *Achyrocline satureioides* products, no studies concerning its stability have been reported.

In this context, the present work was designed to evaluate the thermal and photo stability of an *Achyrocline satureioides* spray dried powder (SDP40) where the main polyphenols were selected as reference substances, quercetin, luteolin, 3-*O*-methylquercetin and caffeic acid.

2. Investigations, results and discussion

2.1. Thermal stability assay

2.1.1. Long term testing

This test was carried out under room-controlled temperature, simulating storage conditions.

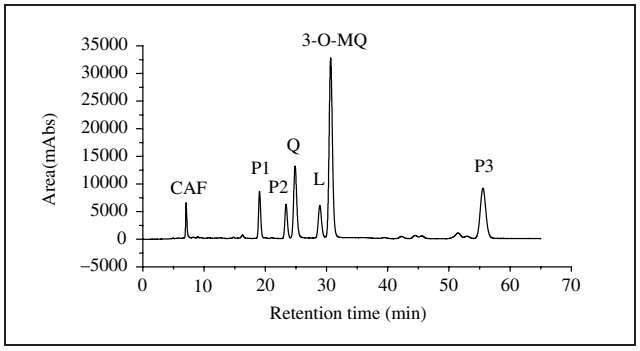

Fig. 1: Fingerprint chromatogram from SDP40

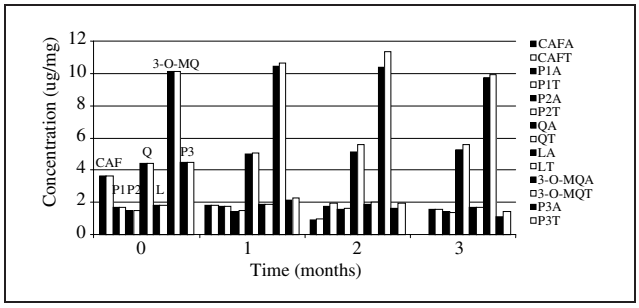

Fig. 3: Polyphenol concentration in SDP40 under accelerated conditions (50 °C/90% RH), A = amber flask and T = transparent flask

Samples of SDP40 (1 g) were stored in amber or transparent glass flasks. They were analyzed after 0, 3, 6, 9 and 12 months.

The LC profile of the SDP40 at the time zero is shown in Fig. 1, where the presence of the three major polyphenols quercetin (Rt = 25.68), 3-*O*-methylquercetin (Rt = 31.87) and a non-identified peak P3 (Rt = 58.82) can be observed. Luteolin (Rt = 29.91), caffeic acid (Rt = 7.14) and two non-identified compounds P1 (Rt = 19.60) and P2 (Rt = 24.21) are also present in lower concentration than the first ones.

The sensorial characteristics of the SDP40 did not change during all the test period (12 months), yellow fine powder and the peculiar smell. The loss on drying of the samples, measured before the polyphenol assay, were in the range from 4.82% to 5.85%.

For herbal medicines with unspecified active compounds, the stability of the product is evaluated by quantifying a group of compounds. In this case, variations of ±10% from the initial content is stated as acceptable (Hefendehl 1987; EMEA 2001). For *Achyrocline satureioides,* considering that the polyphenols play an important role in the antiinflamatory (Simões et al. 1988) and also in the antioxidant activities (Polydoro et al. 2004), these constituents were selected for evaluating the stability of SDP40. Moreover, the low polyphenol stability is an additional reason for selecting this class of compounds as indicator of stability. The SDP40, submitted to long term testing, demonstrated good stability regarding the total polyphenols, which represent the sum of the four main constituents, quercetin, luteolin, 3-*O*-methylquercetin and caffeic acid. Figure 2 shows that the polyphenol content remained within an acceptable range for 9 months (±10%). No significant differences were observed in the total polyphenol content among samples conditioned in amber or transparent flasks, denoting that, in solid state, the polyphenol fraction remained stable under both storage conditions.

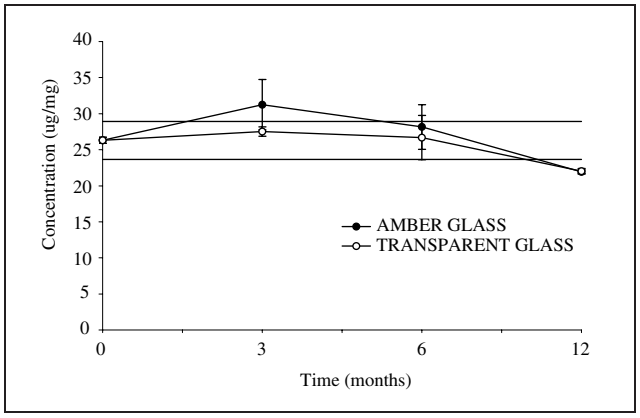

Fig. 2: Total polyphenol concentration in SDP40 (25 °C/60% RH) (caffeic acid, P1, P2, quercetin, luteolin, 3-O-methylquercetin and P3). Lines represent 10% of the onset value

2.1.2. Accelerated term testing

This test was carried out according to the Brazilian official requirements for drugs (ANVISA/BR 2002). Temperature at 50 °C and 90% relative humidity were the conditions employed during the 3 months test; a climatic chamber (Nova Ética mod. 420 CLD) was used to obtain these conditions. Samples of SDP40 (1 g) were stored in each amber or transparent flask containers and analyzed after 0, 1, 2 and 3 months.

Significant changes in sensorial properties of SDP40 were observed during the test. After 3 months the SDP40 samples became brown and smelling acetic. The residual humidity improved from 4.42% to 7.47% in the samples conditioned in amber flasks and from 4.42% to 5.57% for the samples maintained in transparent flask containers.

The variation of the polyphenol content during the period of 3 months is shown in Fig. 3. Significant changes can be observed at this condition, especially regarding the caffeic acid and compound P3 contents. After three months, caffeic acid is not detected anymore and the compound P3 had its content reduced to 25.3% (amber flask) and 31.5% (transparent flask), from its initial content. By the other hand, after 3 months, the content of quercetin improved from 4.4 ± 0.1 μg/mg to 5.3 ± 0.1 μg/mg when conditioned in amber flask and from 4.4 ± 0.1 μg/mg to 5.6 ± 0.1 μg/mg when conditioned in transparent flask container. These increase in quercetin content corresponds, respectively, to 120% and 127% of the initial concentration. The explanation for this phenomenon is sill nuclear, however, the hypothesis of conversion of P3 in quercetin was based on the simultaneous decrease in P3 concentrations, during the first month. Although this hypothesis cannot completely ruled out, the observation that, after two months, the content of P3 continued decreasing without the corresponding increase in quercetin concentration did not corroborate this hypothesis. The hypothesis of hydrolysis of a quercetin conjugated form, as quercetin glycoside, can be also formulated since it is common in herbal medicinal products (Heigl and Franz 2004). Investigations in view to enlighten this phenomenon are running in our laboratory.

2.1.3. Stress testing

A temperature of 80 °C was chosen for evaluating the changes that could be occurring in the polyphenol consti-

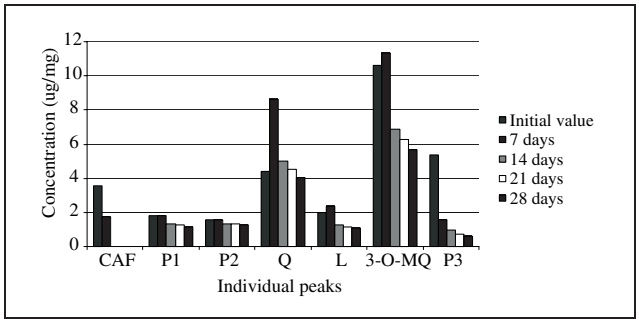

Fig. 4: Polyphenol concentration in SDP40 under stress conditions in unprotected open-dishes (80 °C/28 days)

tuents present in SDP40, specially the quercetin, luteolin, 3-*O*-methylquercetin, caffeic acid and also with P3, under stress conditions. The SDP40 samples (1 g) were conditioned in unprotected open-dishes. The results of polyphenol content, after 28 days, are presented in Fig. 4.

After day seven the fine and yellow powder became brown to deep brown and presented a smell characteristic of sugar degradation which became acetic later. The SDP40 residual humidity was reduced from 4.8% to 0.5%.

At the first seven days at 80 °C, we did observe a significant increase in the quercetin (Q) concentration (almost 2 fold), from 4.4 ± 0.1 μg/mg to 8.6 ± 0.1 μg/mg. After 14 days the quercetin concentration decreased, reaching the initial value. In contrast the caffeic acid content showed a significant decrease during the first seven days, from 3.5 ± 0.1 μg/mg to 1.2 ± 0.1 μg/mg disappearing, totally, after fourteen days. Also P3 decreased in its concentration from 5.4 ± 0.07 μg/mg to 0.5 ± 0.03 μg/mg, after 28 days.

Luteolin and 3-*O*-methylquercetin presented an intermediate stability, showing decreases of 46.5% and 46.8% respectively at the end of the test period. P1 and P2 were the less unstable constituents showing decreases, of 36.7% and 21.0% respectively, after 28 days 80 °C exposure in unprotected open-dishes.

No degradation peaks appeared in the fingerprint chromatogram during 28 days.

In view to determine, more precisely, what time the quercetin content increase, samples of SDP40 were exposed to stress conditions, 80 °C for a week and quercetin was determined at 2, 5 and 7 days. The results presented in Fig. 5 demonstrate that at 48 hours exposure, the highest amount of quercetin was detected, from 4.09 ± 0.13 μg/mg ($t = 0$) to 6.55 ± 0.05 μg/mg ($t = 48$ h), about 160% of the initial SDP40 quercetin concentration.

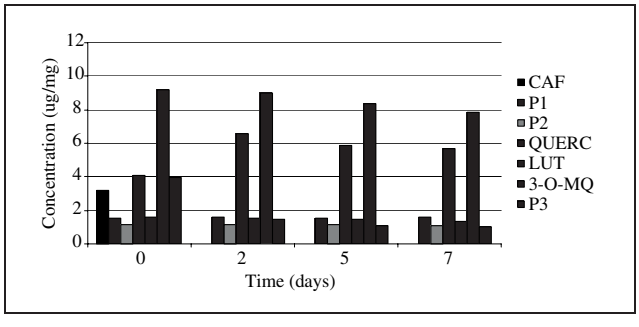

Fig. 5: Polyphenol concentration of SDP40 under stress conditions in unprotected open-dishes (80 °C/7 days)

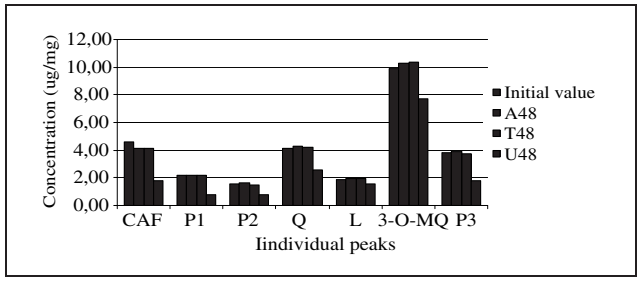

Fig. 6: Polyphenol concentration in SDP40 submitted to UV-C radiation (Light express LE UV, 254 nm, 30 W) initial value and after exposure in A = amber flask, T = transparent flask and U = unprotected for 48 h

2.2. Photo stability assay

The samples were submitted to UV-C radiation (Light express LE UV, 254 nm, 30W) in order to detect the stability of the polyphenol presented in SDP40, conditioned in different containers: amber flask, transparent flask and open-dishes.

The sensorial characteristics of the SDP40 did not change during all the experiment period, maintaining its original properties. The loss on drying of the samples, measured before the polyphenol assay, was in the range of 4.71% to 4.87% for the samples conditioned in amber flasks; 4.71% to 4.57%, for the samples contained in the transparent flasks and 4.71% to 2.77% in open-dishes denoting that no significant differences were provided by amber or transparent flasks. In contrast the samples conditioned in open-dishes presented reduction of the humidity content after 48 h of exposure, reaching a value of 2.77%.

The polyphenol content in SDP40, when samples were conditioned in amber or transparent flasks, presented stable behavior during the time of the experiments, 48 h (Fig. 6). However, when SDP 40 was stored in open-dishes the polyphenol concentration showed a significant decrease in concentration, especially for quercetin, 3-*O*-methylquercetin, caffeic acid (Fig. 7), P1, P2 and P3 (Fig. 6). Luteolin showed the highest stability under UV light radiation.

Taken together, the results of photo stability tests demonstrated that the SDP40 shows sensitivity against UV light radiation when conditioned in open-dish, while amber and transparent flasks demonstrated efficiency to protect the SDP40, during 48 hours of light exposure.

In conclusion, based on the total polyphenol content, SDP40 shows to be stable for nine months, when conditioned in amber or transparent flasks at storage conditions of 25 °C of temperature and 60% relative humidity. Similar behavior was observed in the photo stability assay, where we observed good stability of SDP40 conditioned in amber or transparent flasks against UV-C radiation (Light express LE UV, 254 nm, 30W) for 48 h. We also demonstrated that SDP40, despite being a solid product, is unstable against high temperature 80 °C and UV-light when it was stored in unprotected open-dishes. The LC polyphenol analysis demonstrated also that the behavior of individual constituents have still to be better enlightened, especially the peak in quercetin content after 1 month at 50 °C or 2 days at 80 °C exposition, respectively.

3. Experimental

3.1. Chemicals

The following chemicals were used: methanol for liquid chromatography (LC grade, Merck, Darmstadt, Germany) and phosphoric acid (Merck,

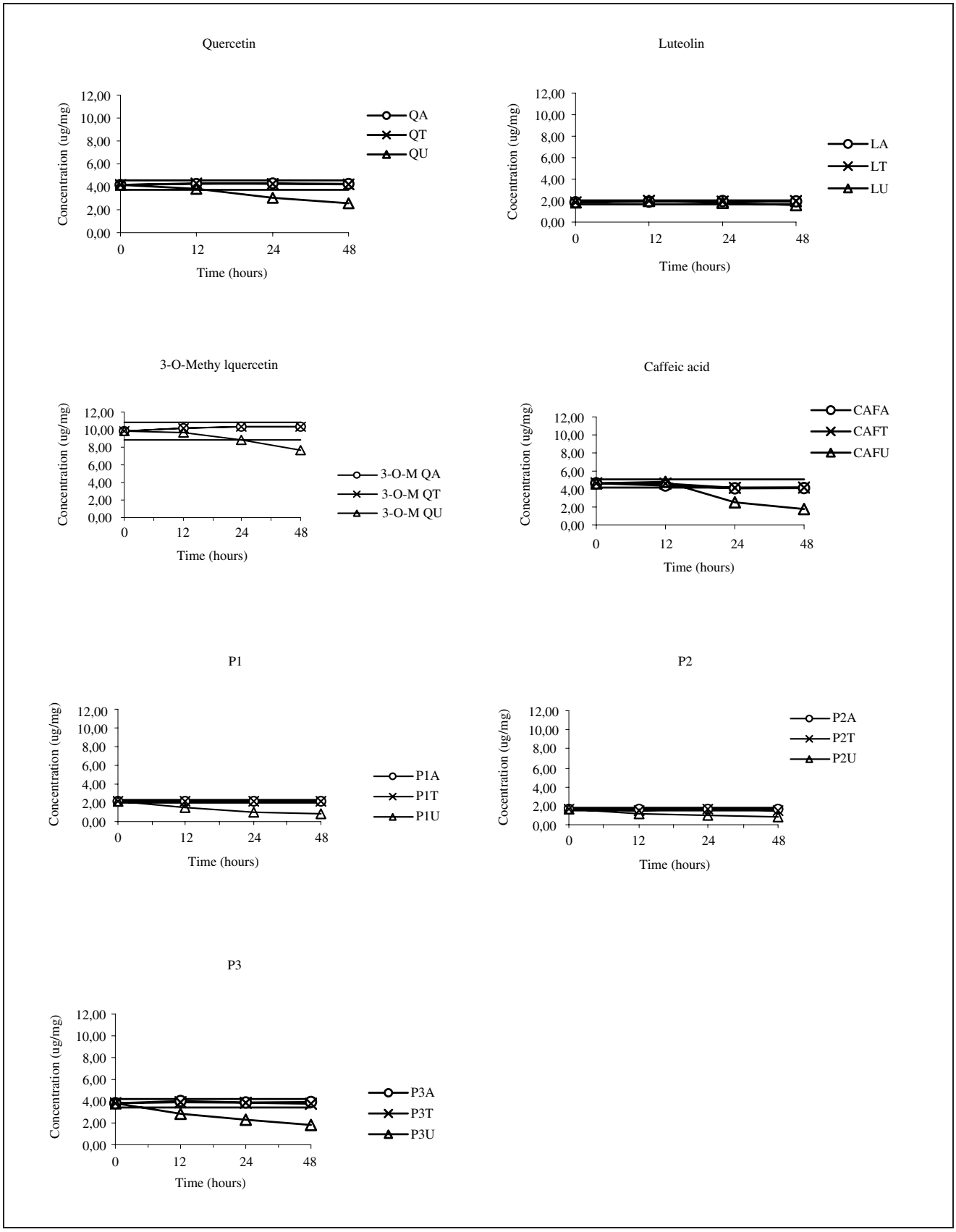

Fig. 7: Polyphenol content in SDP40 samples submitted to UV-C radition (Light express LE UV, 254 nm, 30 W) A = amber flask, T = transparent flask and U = unprotected. Lines represent 10% of the onset value

Darmstadt, Germany), water (Milli-Q system, Millipore, Bedford, MA, USA), polysorbate 80 (Delaware, Porto Alegre, Brazil), luteolin (Sigma, St. Louis, MO, USA), caffeic acid (Sigma, St. Louis, MO, USA) quercetin (Merck, Darmstadt, Germany) and colloidal silicon dioxide (CSD) (Degussa, Düsseldorf, Germany).

3.2. Preparation of Achyrocline satureioides spray dried powder (SDP40)

The spray-dried powder was prepared following the procedure described in the brazilian patent (Bassani et al. 2001). Briefly, inflorescences of *Achyrocline satureioides* Lam. (DC), Asteraceae, were extracted by maceration, with ethanol 40% (v/v) in order to obtain the extractive solution. The ethanol content was reduced to 10% (v/v), colloidal silicon was added then, the dispersion was dried, using a Production Minor Spray Dryer plant – NIRO A/S, under the following operating conditions: inlet/outlet temperature 180/110 °C; 10.400 rpm and feed rate 130 mL/min. The SDP40 contained 50% of *A. satureioides* dry residue and 50% of solid excipients.

3.3. LC analysis

3.3.1. Sample preparation

The SDP40 (0.5 g) was extracted with ethyl acetate for two hours under magnetic stirring. The supernatant was filtered through a filter paper (grade 1 : 11 μm, Whatman, UK) and the volume was made up to a 50 ml with the same solvent. An aliquot of 25.0 mL was evaporated, the residue was dissolved in methanol and transferred to a 25.0 mL volumetric flask. From this solution an aliquot of 2.0 mL was diluted to 20.0 mL with methanol 53% (v/v). This solution was filtered through 0.45 μm membrane filter (HVHP-Millipore) and analyzed by LC (De Souza 2002). This procedure was repeated three times for each sample, moreover each point was injected three times in the LC equipment.

3.3.2. Standard curves

Quercetin and luteolin were used as external standards; they were dissolved in a mixture of methanol : water (53 : 47, v/v) yielding concentrations of 0.1; 0.2; 0.3; 0.4; 0.5 and 10.0 μg/mL. The linear equations ($n = 6$) were $y = 115873x - 27266$ ($r = 0.9991$), $y = 122605x - 13196$ ($r = 0.9998$) and $y = 28771x - 2715{,}09$ ($r = 0{,}9999$) for quercetin, luteolin and caffeic acid, respectively.

3.3.3. Polyphenol assay in the samples

The content of quercetin in the SDP40 samples and that corresponding to the compound P3 were calculated by the quercetin linear equation. The content of caffeic acid by the corresponding equation and the concentrations of luteolin, 3-*O*-methylquercetin, compounds P1 and P2 were calculated by the linear equation of luteolin. Total polyphenol content in the samples was obtained by the sum of the content of the quercetin, luteolin, 3-*O*-methylquercetin and caffeic acid.

3.3.4. Chromatographic conditions

The quantitative analysis was carried out according the method described by De Souza et al. (2002). A Shimadzu liquid chromatograph equipped with a LC-10 AD pump, an auto sampler SIL-10 A and UV-vis detector SPD-10 A controlled by CLASS LC-10 software were used. The column was a Shim-pack CLC-ODS (M) RP-18, 5 μm, 250 × 4 mm i.d. and a pre-column Waters (10 × 4 mm i.d.) packed with Bondapack C-18 10 μm (Waters, Milford, USA) was employed. The mobile phase consisted of a mixture, methanol : phosphoric acid 0.16 M (53 : 47, v/v), filtered through a 0.45 μm membrane filter (Millipore-HVHP). The flow rate was 0.6 mL/min; the injection volume was 20 μL and the peaks were detected at 362 nm. In order to check the peak purity of each compound Waters Millenium DAD was used. The absorbance was measured from 200 to 800 nm every 1 s with 4.8 nm resolution.

3.4. SDP40 thermal stability

The thermal stability of SDP40 was carried out in triplicate at three different temperatures and relative humidity (RH) conditions:

- 25 °C ± 2 (60% RH ± 5), for 12 months (long term testing); this condition was obtained in a room-controlled temperature, documented by manual measurement of temperature and humidity. The samples were conditioned in amber or transparent glass flasks. The SDP40 LC analyses were carried out 0, 3, 6, 9 and 12 months.
- 50 °C ± 2 (90% RH ± 5), for 3 months (accelerated testing); this condition was obtained in a climatic chamber (Nova Ética mod. 420 CLD). The samples were conditioned in amber or transparent glass flasks. The SDP40 LC analyses were carried out at 0, 1, 2 and 3 months.
- 80 °C ± 2, for 28 days (stress testing); a conventional drying oven was used for obtaining this condition (Biomatic mod. 1305). The samples were conditioned in unprotected open-dishes. The SDP40 LC analyses were carried out at 0, 7, 14, 21 and 28 days.

3.5. SDP40 photo stability

The photo stability of SDP40 was performed in triplicate at three different storage conditions: amber flasks, transparent flasks or open-dishes.
The samples were submitted to UV-C radiation (Light express LE UV, 254 nm, 30W) in light chamber, with internal cover by mirror. The LC analyses were carried out at 0, 12, 24 and 48 h.
Samples of SDP40 (1 g) was stored in each amber or transparent tight flask containers, or also, in unprotected open-dishes. The polyphenol content was determined in all of samples by LC method, taking into account the residual humidity after stability tests.

3.6. Loss on drying

Values of loss on drying were obtained by gravimetric method, according to the Brazilian Pharmacopoeia (Farm. Bras. 1988).

3.7. Sensorial characteristics

The samples from thermal and photo stability tests were analyzed by their characteristics of smell, color and appearance of the powder, in all times, before the LC analysis.

3.8. Abbreviations

P1, P2 and P3 not identified peaks, CAF = Caffeic acid, Q = Quercetin, L = Luteolin, 3-O-MQ = 3-*O*-methylquercetin, A = amber flask, T = transparent flask, U = unprotected.

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